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VOLUME 27 NUMBER 6

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC AND HISTOCHEMISTRY

NOVEMBER 1952

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STAIN TECHNOLOGY

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THE USE OF LEAD TETRAACETATE, BENZIDINE, O-DIANISIDINE AND A "FILM TEST" IN INVESTIGATING THE PERIODIC-ACID-SCHIFF TECHNIC

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Received for publication Apr. 29, 1952

ABSTRACT.—**In order to obtain a better understanding of the "periodic-acid-Schiff" reaction, also known as the "periodic-acid: fuchsin-sulfurous-acid" reaction, three types of investigations were carried out:**

1) The Schiff reagent was replaced by other aldehyde reagents: benzidine or o-dianisidine. There was no significant change in the histological distribution and intensity of the reactions occurring after periodic acid oxidation.

2) Periodic acid was replaced by another oxidizing agent: lead tetraacetate (dissolved in acetic acid). There was no significant change in the histological distribution of the reactions with the Schiff reagent, but some change in their intensity. It was concluded that 1,2–glycols and α-amino alcohols play the main role in the reactions with both oxidants. The presence of α-hydroxy acids in some types of mucous cells is suggested by the results with lead tetraacetate.

Incidently, glycogen and starch are not sufficiently oxidized by lead tetraacetate (in acetic acid) at room temperature to give positive reactions with the Schiff reagent, while cellulose and other periodic-acid-Schiff reactive substances are.

3) The staining of films of presumed reactive substances with the periodic-acid-Schiff technic confirms the intense reactivity of many polysaccharides, mucopolysaccharides and mucoproteins, but not of ordinary proteins. (Hyaluronic and chondroitin sulfuric acid are, however, not reactive in vitro).

In conclusion, the periodic-acid-Schiff technic consists of an oxidation of 1,2–glycols and α-amino alcohols to produce aldehyde groups, which are then stained by the Schiff reagent. The "film test" reveals that these radicals are present in certain polysaccharides, mucopolysaccharides and mucoproteins.

Historical

The "periodic-acid-Schiff" technic developed by McManus (1946), Lillie (1947) and Hotchkiss (1948) is generally believed to involve two chemical reactions: (1) an oxidation of 1,2–glycol and α-amino alcohol groups with periodic acid to produce aldehyde groups; and (2) a reaction of these aldehyde groups with the Schiff reagent to produce a violet color. The reactions observed in histological sections with this technic were attributed by Hotchkiss (1948) to the presence of polysaccharides, free or combined with proteins. The present work was carried out to examine the validity of this interpretation.

It was decided to examine first the validity of the second step of the reaction, that is, to see whether, under the conditions of the technic, the substances recolorizing the Schiff reagent contained aldehyde groups. It had indeed been proposed that many substances other than aldehydes were able to recolorize the Schiff reagent (Wieland and Scheuing, 1921; Lison, 1932, 1936; Stedman and Stedman, 1943a and b,1944). The periodic-acid-Schiff technic was, therefore, modified by replacing the Schiff reagent with benzidine or o-dianisidine, both of which are known to react with aldehydes to produce colored Schiff bases by a simple condensation reaction (van Eck, 1923; Wasicky and Frehden, 1937; Feigl, 1946; Karrer, 1946). The results were then compared with those obtained with the standard periodic-acid-Schiff method.

The presence of aldehydes in periodic-acid-treated slides was thus demonstrated.

It was then necessary to examine the significance of the first step in the technic, that is, to see whether the aldehydes were due to oxidation of 1,2–glycol and α-amino alcohol groups. Jeanloz (1950) questioned this since hyaluronic acid (which he believes yields a strong reaction when present in sections) consumes only a very small amount of periodic acid *in vitro*. Wolman (1950) claimed that unsaturated lipids give positive reactions, due to the oxidation of the ethylenic linkage by periodic acid to yield aldehydes. In order to examine whether or not 1,2–glycol and α-amino alcohol groups had reacted with periodic acid, the reaction was carried out with lead tetraacetate – a reagent with similar, though not identical oxidative properties (Criegee, 1931, 1940, 1948; Heidt, Gladding and Purves, 1945). The results were again compared with those obtained with the standard periodic-acid-Schiff method.

Finally, Hotchkiss' hypothesis that polysaccharides were responsible for the reaction had to be reexamined, since Hotchkiss himself (1948) showed that a spot test devised to identify reactive substances revealed positive reactions with many proteins. However, the spot test is not comparable to the histological technic, since it retains all

reagents as well as the products of periodic acid oxidation in the reaction mixture, whereas reagents and soluble reaction products may be washed out of histological sections. For this reason, a "film test" was developed in which thin films of material were placed on slides and stained by the periodic-acid-Schiff technic. The reactivity of a number of proteins, polysaccharides, mucopolysaccharides and mucoproteins were ascertained by this film test. On the whole, the significance of the periodic-acid-Schiff technic was clarified by these experiments.

Methods

The histological sections used in the experiments to be described were obtained from the following organs of adult male rats: liver, kidney, stomach, duodenum, colon, trachea, thyroid, salivary glands, testis, epididymis, prostate and eye. Fixation was in Orth fluid, dehydration, in dioxane, and embedding, in paraffin.

I. TREATMENT OF SECTIONS WITH PERIODIC-ACID-SCHIFF

The method used was essentially similar to those described by Lillie (1947), McManus (1948) and Hotchkiss (1948).

Solutions:

1. Periodic acid. Periodic acid, 0.8 g., was dissolved in 20 ml. of distilled water, and 10 ml. of 0.2 *M* sodium acetate and 70 ml. of ethyl alcohol were added.

2. Schiff reagent. Basic fuchsin, 2 g., was dissolved in 400 ml. of boiling water, cooled to 50° C. and filtered. Ten milliliters of 2*N* hydrochloric acid, then 4 g. of potassium metabisulfite were added to the filtrate and the mixture was left in the dark overnight at 4° C. One gram of decolorizing charcoal was added, and, after mixing for 2 minutes, the solution was filtered. About 12 ml. of 2*N* HCl was added in small portions until the mixture did not become pink when a few drops were allowed to dry on a glass slide.

3. Sulfite water. Potassium metabisulfite, 0.4 g., was dissolved in 100 ml. of distilled water to which 1 ml. of concentrated hydrochloric acid had been added.

Procedure:

1. Oxidize the deparaffinized sections in periodic acid for 10 minutes at room temperature.
2. Wash in 70% alcohol for 5 minutes.
3. Stain in Schiff reagent for 45 minutes.
4. Wash in three changes of sulfite water for 5 minutes each.
5. Dehydrate and mount in balsam.

Controls:

Control sections were not oxidized with periodic acid, but were carried through the remainder of the procedure.

Some sections were also treated with saliva at 40° C. prior to the periodic-acid-Schiff technic, in order to distinguish glycogen from other reactive materials.

II. REPLACEMENT OF SCHIFF REAGENT BY BENZIDINE AND O-DIANISIDINE

Solutions:

Benzidine and o-dianisidine were made up as 1% solutions in glacial acetic acid. Both were used in the same manner.

Procedure:

1. Oxidize deparaffinized slides in periodic acid for 10 minutes.
2. Wash in glacial acetic acid for 5 minutes.
3. Stain in benzidine or o-dianisidine reagent for 45 minutes at 50° C.
4. Wash in glacial acetic acid for 5 minutes.
5. Wash in distilled water for 5 minutes, then dehydrate and mount in balsam.

Controls:

The control slides were not treated with periodic acid.

III. REPLACEMENT OF PERIODIC ACID BY LEAD TETRAACETATE[1]

Solution:

One gram of lead tetraacetate was dissolved in 100 ml. of glacial acetic acid. Any brown color which appeared in the solution disappeared on standing.

Procedure:

1. Wash the deparaffinized sections in glacial acetic acid for 5 minutes.
2. Oxidize in lead tetraacetate for *4 hours at room temperature.*
3. Wash in glacial acetic acid for 5 minutes.
4. Wash in distilled water for 5 minutes.
5. Stain in Schiff reagent for 45 minutes, and then wash in three changes of sulfite water as above.
6. Dehydrate and mount in balsam.

---

[1]Lead tetraacetate is now available from Arapahoe Chemicals, Inc., 2800 Pearl Street, Boulder, Colorado, and was also prepared for this work by the method of Fieser (1941).

Lead tetraacetate is known to decompose even when stored in a cool place and protected from light and moisture. However, the reagent remained in good condition after 5 months storage in a "deep freeze". (The amount of lead tetraacetate present in a sample at any time can be determined by iodometric titration according to Dimroth and Schweitzer, 1923).

Like all lead salts, lead tetraacetate is poisonous and should be handled with care, so as to avoid contact with the skin.

Controls:

Deparaffinized sections were left in glacial acetic acid for 4 hours prior to step 4.

In another series of experiments, the lead tetraacetate oxidation was carried out for varying periods of time at 40°, 50°, 60°, and 70° respectively, instead of 4 hours at room temperature. The other conditions were maintained as described above.

IV. THE PREPARATION AND STAINING OF FILMS FOR THE "FILM TEST"[2]

One percent aqueous solutions of the various substances to be tested for periodic-acid-Schiff reactivity were prepared and centrifuged to remove suspended particles. An area of approximately 2.5 × 5.0 cm. was marked off on a microscope slide which was then washed with chloroform-ethanol and weighed. A 0.5 ml. portion of the solution was placed on the slide and spread with the tip of the pipette over the outlined area, then allowed to dry at 45° C. on a level hot plate. The process was repeated with another 0.5 ml. of solution and the slide weighed again. The amount of dry material on each slide was adjusted to approximately 10 mg. Assuming a density of 1.3 for the substances used, the thickness of the film was calculated to be approximately 6 μ, and was therefore comparable to that of a histological tissue section. In the case of glycogen, the amount of material on the slide had to be reduced to 1.3 mg. to obtain a suitable reading on the densitometer. This represented about one-eighth of the material on the other slides, that is to say, a film less than 1 μ thick.

The films were fixed in 70% alcohol for 10 minutes and then stained by the periodic-acid-Schiff method as above. Controls were run in the same way, omitting the periodic acid oxidation.

The films were examined microscopically and the intensity of their purple color estimated visually (Table 2). Later an attempt was made to obtain an objective estimate of the reactivity of the various substances by measuring the density of the films stained with periodic-acid-Schiff and their controls with the aid of the Photovolt densitometer. A microphotometric set up (8× ocular and 3.5× objective) was used to project the image of the films on a frosted glass. The phototube was placed facing a defined area of the frosted glass. A green filter was placed between the source of light and the film to obtain maximum absorption. Six readings were taken in different areas on the films. The densities were then calculated using the formula $D = \log I - \log T$, where I represents the reading obtained on the control, and T that on the stained film (Table 2).

[2] A preliminary description of this technic was reported by Glegg, Clermont and Leblond (1951).

TABLE 1. CLASSIFICATION OF STRUCTURES ACCORDING TO THEIR RESPONSES TO THE SCHIFF REAGENT, BENZIDINE OR O-DIANISIDINE AFTER PERIODIC ACID OR LEAD TETRAACETATE OXIDATION

	1	2	3	4	5
			Lead-tetraacetate-Schiff		
	HIO_4-Schiff	HIO_4-benzidine (or HIO_4-dianisidine)	(4 hours oxidation, room temp.)	(1 hour oxidation at 50°C.)	(3 hours oxidation at 70°C.)
I *Connective tissue derivatives*					
reticulin material					
reticulin fibers, stomach	++	++	+	+	0
basement membranes, kidney	+++	++	+	±	0
trachea	++	++	+	+	0
lens capsule	+++	+++	++	++	0
cartilage matrix, central portion	+	+	+	++	+++
chrondrogenic zone	++	++	++	++	0
II *Cell membranes and derivatives*					
membrane of cells of potato tuber	++		+++	+++	+++
brush border, kidney	++	+	+	±	0
striated border, duodenum	+++	+++	+++	+++	++

III *Secretion products*					
exocrine					
mucous secretion					
of stomach, surface epithelium	+++	+++	+++	+++	++
mucous neck cells	++	++	+++	+++	++
of duodenum, goblet cells	+++	+++	+++	+++	++
Brunner's glands	++	++	++	+++	++
of colon, upper goblet cells	+++	+++	+++	+++	++
deeper goblet cells	+	+	+++	+++	++
of submaxillary acini	++	++	++	++	0
sero-mucoid granules					
acidophilic: serous tubules, submaxillary	++	++	+	±	0
neutral: glandular cells of epididymis	+++	++	++	+	0
other secretions: prostate gland	+++	++	++	+	0
coagulating gland	+++	++	++	+	+
endocrine:					
thyroid colloid	+++	+++	+++	+	0
other cell products:					
acrosomic system of spermatids, acrosome	+++	+++	++	+	0
head cap	++	++	+	+	0
glycogen (liver, muscle, cartilage cell)	+++	+++	0	+	+++
starch (plant cells)	+++		0	±	++

The same solutions used for the preparation of the films were also subjected to the *spot test* as described by Hotchkiss (1948) (Table 2).

TABLE 2. A COMPARISON OF THE HOTCHKISS SPOT TEST AND THE FILM TEST

Reacting material	Spot test rating	Film test	
		Visual rating	Optical density
Casein	+	−	0.12
Pectinase	+	−	0.01
Trypsin	++	−	0
Pepsin	++	−	0.22
Edestin	++	−	0.16
Lipase	++	−	0.01
Hyaluronidase	++	−	0.09
*Hyaluronic acid (a)	++	−	0
(b)	++	−	0
(c)	++	−	0
Gelatin	−	−	0
Rennin	−	−	0.06
Urease	+	+	
Serum globulin	++	+	
Egg albumen	++	++	0.36
Thyroglobulin	++	++++	1.17
Gastric mucin	++	+++++	1.76
Pectin	++	+++++	1.86
Glycogen	++	+++++	1.58†

*Hyaluronic acid (a) and (b) were two different samples from human umbilical cord; (c) was from bovine vitreous humor.

†1.3 mg. per slide.

Results

REACTION SITES WITH THE PERIODIC-ACID-SCHIFF TECHNIC

The observed reactions are summarized in the first column of Table 1. Many of them were similar to those previously reported by Leblond (1950), but a few new findings will be described within the framework of the classification of this author: (1) *Connective tissue derivatives.* In cartilage, there is a definite reaction of the thin chondrogenic zone, present along the edges, with a weak reaction of the matrix itself (Fig. 15). There is an intense staining of reticulin fibers and basement membranes (Table 1). (2) *Cell membrane derivatives:* In the potato tuber the cell membrane gave a strong reaction (Fig. 12). (3) *Secretion products.* There were 2 types of reactions of *mucous cells.* Thus, the mucous cells of the surface epithelium of the stomach gave more intense reactions than the mucous neck cells. Similarly, the goblet cells of the duodenum reacted more strongly than the mucous cells of Brunner's glands (Fig. 1). The goblet cells in the upper two-thirds of the crypts in the colon reacted much more intensely than those cells in the lower one-third of the crypts, a fact already reported by Lillie (1949), (Fig. 5). Furthermore,

the more intense of these reactions of stomach, duodenum and colon were characterized by a deep violet color, whereas the less intense ones were pink. To the list of *seromucous cells* previously described, the glandular cells found in the epididymis may be added (Fig. 7). The seromucous cells may be classified according to whether they give acidophilic reactions (serous tubules of submaxillary) or neutral reactions (parotid acini, glandular cells of epididymis).

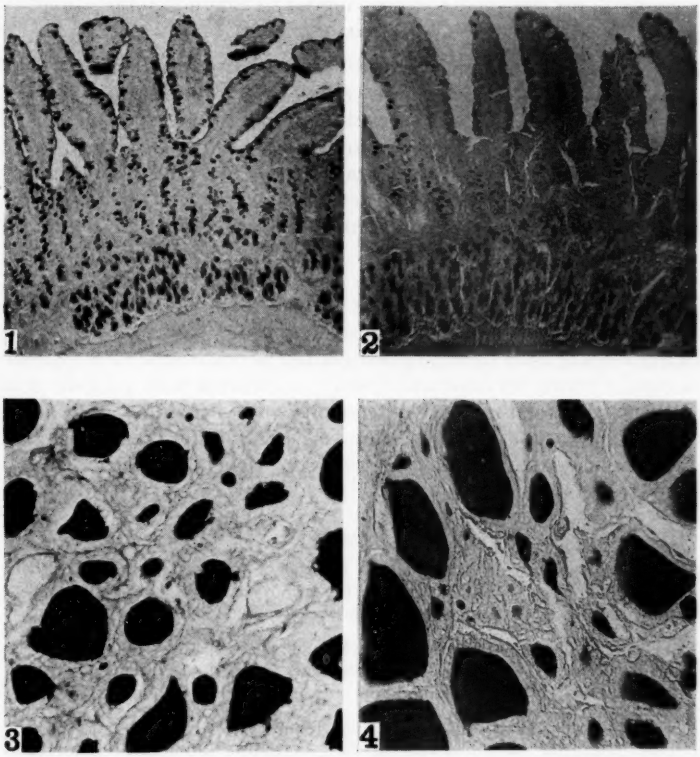


PLATE 1

Tissues fixed in Orth's fluid, oxidized by periodic acid, and stained with either the Schiff reagent (left) or o-dianisidine (right). The Schiff stained tissues were photographed with a green filter, and the dianisidine stained tissues with a blue filter.

FIG. 1 and 2 — Duodenum. The mucous secretion of goblet cells and Brunner's glands is stained in the same manner with periodic-acid-Schiff (left) or periodic-acid-dianisidine (right).

FIG. 3 and 4 — Thyroid. The colloid stains with both technics.

Finally, it was found convenient to list various other materials as secretion products, namely, the acrosomic system of spermatids (Leblond and Clermont, 1952), glycogen (Fig. 9), and finally, the starch granules found in sections of potato tuber (Fig. 12). The reactions of glycogen and starch are prevented by treatment of the sections with saliva.

REPLACEMENT OF THE SCHIFF REAGENT BY BENZIDINE OR O-DIANISIDINE: THE PERIODIC-ACID–BENZIDINE AND PERIODIC-ACID–DIANISIDINE TECHNICS

The results with either benzidine or o-dianisidine after periodic acid oxidation were brownish yellow colorations located in sites (Table 1, column 2) identical to those of the purple reaction sites of periodic-acid-Schiff (Table 1, column 1). The relative intensities of the reactions were almost identical to those observed with periodic-acid-Schiff (Fig. 1–4). Thus, the replacement of the Schiff reagent by either of these 2 aldehyde reagents resulted in the staining of all the recognized reaction sites.

The only difference found was an intense staining of the crystalline fibers in the lens of the eye with periodic-acid–benzidine and periodic-acid–dianisidine. This structure did not stain with the Schiff reagent after oxidation with either periodic acid or lead tetraacetate. Control sections which were not treated with periodic acid showed completely negative reactions with benzidine and dianisidine, indicating that the observed periodic-acid–benzidine and periodic-acid–dianisidine reactions were dependent on prior oxidation with periodic acid.

PLATE 2

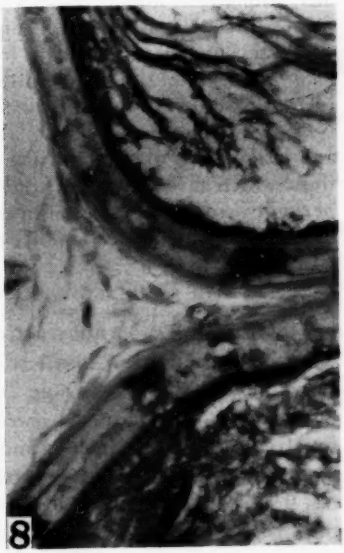
Tissue fixed in Orth's fluid. Stained with Schiff reagent after oxidation with periodic acid (left), or lead tetraacetate for 4 hours at *room temperature* (right). The tissues were photographed with a green filter.

FIG. 5 — Colon. Periodic-acid-Schiff technic. The mucous secretion of the goblet cells in the upper two-thirds of the crypts shows an intense reaction. In the lower third of the crypts the goblet cells show only a very weak reaction. × 100

FIG. 6 — Colon. Lead-tetraacetate-Schiff technic. All the goblet cells stain intensely. Note that the goblet cells in the lower third of the crypts stain much more intensely than with periodic-acid-Schiff (compare Fig. 5). × 100

FIG. 7— Section through the walls of the tubules of the epididymis situated in the caudal region of this organ. Periodic-acid-Schiff technic. Some rectangular, non-ciliated cells in the epithelial lining of the tubules, or "seromucoid" cells, are intensely stained. The ciliated columnar cells contain some minute reactive granules in the Golgi region. The cilia also show a moderate reaction. × 350

FIG. 8 — Same section of epididymis as in Fig. 7. Lead-tetraacetate-Schiff technic. The "seromucoid" cells of the epithelium stain less intensely than in the periodic-acid-Schiff treated section. The Golgi granules are faintly visible, but the cilia show the same degree of reactivity as in Fig. 7. × 350



REPLACEMENT OF PERIODIC ACID BY LEAD TETRAACETATE: THE LEAD-TETRAACETATE-SCHIFF TECHNIC

This technic gave color reactions on tissue sections which varied with the experimental conditions of oxidation (Table 1, columns 3, 4 and 5). Oxidation for 4 hours at room temperature (column 3) was found to be the most satisfactory condition for obtaining maximum intensity of most of the structures at the same time (with the exception of glycogen and starch which did not stain, Fig. 10 and 13) and is therefore considered as the most suitable standard procedure. Oxidation for 1 hour at 50° C. (column 4) caused a decrease in intensity of the staining for many structures, although glycogen and starch now stained. More drastic oxidation for 3 hours at 70° C. (column 5) caused a decrease or disappearance of all reactions, except glycogen and starch (Fig. 11 and 14) and the central portion of the cartilage matrix (Fig. 18), which stained more intensely.

Under the standard conditions (4 hours oxidation at room temperature) there were three types of reaction sites (Glegg and Clermont, 1952) as evaluated by the intensity of Schiff staining after periodic acid or lead tetraacetate oxidation: (a) those which gave an *equivalent response with both lead tetraacetate and periodic acid:* the striated border of the duodenal epithelium; many types of mucous cells such as submaxillary acini, surface epithelium of the stomach, goblet cells and Brunner's glands of the duodenum and superficial goblet cells of the colon (Fig. 5 and 6); and the thyroid colloid; (b) those which gave a *stronger response with periodic acid than with lead tetraacetate:* the brush border of the kidney, the reticulin structures, the serous tubules of the submaxillary gland, the seromucoid cells of the epididymis (Fig. 7 and 8), the secretions of the prostate and coagulating glands, and the acrosomic system of spermatids; (c) those which gave a *stronger response with lead tetraacetate than with periodic acid:* many types of mucous cells such as mucous neck cells of the stomach and deep goblet cells of the colon (Fig. 5 and 6).

THE STAINING OF FILMS OF VARIOUS SUBSTANCES

The periodic-acid-Schiff reactivity of a large number of substances was examined by the "film test" described under methods and by the Hotchkiss spot test (Table 2). For the first 8 substances listed, a discrepancy was obtained between the two methods, which always involved a negative film test and a positive spot test. On the other hand, agreement between the two methods was obtained with the last 9 substances listed. It is believed that the use of films duplicates the histological procedure and provides a more dependable basis than the spot test on which to predict the reactivity of various substances in tissue sections.

The film test, therefore, indicated that the proteins casein, pectinase, trypsin, pepsin, edestin, lipase, hyaluronidase, gelatin and rennin gave negligible periodic-acid-Schiff reactions. Weak positive reactions were obtained with urease and serum globulin and a stronger reaction with egg albumen. Very strong reactions were obtained with thyroglobulin, gastric mucin, pectin and glycogen. Three different samples of pure hyaluronic acid gave negative results.

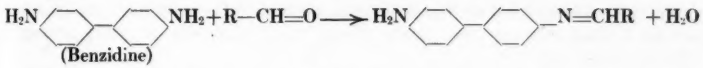
Discussion

This investigation was designed to study the significance of the periodic-acid-Schiff technic. As a preliminary step, the specificity of the Schiff reagent for aldehyde groups was investigated by comparing the results with those obtained using two other aldehyde reagents, namely, benzidine and o-dianisidine. The next step was to examine how aldehydes are produced by periodic acid. Information on the reactive radicals was obtained by replacing periodic acid with lead tetraacetate. Finally, an attempt was made to identify the substances containing such radicals.

THE NATURE OF THE FUNCTIONAL GROUPS REACTING WITH THE SCHIFF REAGENT IN SECTIONS TREATED WITH PERIODIC ACID

The Schiff reagent is known to give a strong color with aldehydes, but according to Lison (1932, 1936) it can also be recolorized by free alkalis, organic bases, some hydrolysable salts, ketones, and ethylenic linkages. To make sure that the reactions observed with the Schiff reagent in periodic acid treated sections were due to aldehydes, the results were compared with those obtained using other aldehyde reagents.

It had already been shown that dinitrophenylhydrazine stained some tissues oxidized with periodic acid in the same manner as the Schiff reagent (Monné and Slautterback, 1950). So did Seligman's carbonyl reagent, although glycogen accumulations and the cartilage matrix remained unstained (Herman and Dempsey, 1951). In the present investigation, two reagents for aldehyde groups, namely, benzidine (van Eck, 1923) and o-dianisidine (Feigl, 1946) were used. These are known to react with aldehyde groups as follows:



(Benzidine)

The reactions observed with either benzidine or dianisidine after periodic acid oxidation (Fig. 2 and 4; Table 1, column 2) were in the same locations and of the same relative intensity as those obtained with the Schiff reagent (Fig. 1 and 3; Table 1, column 1), regardless of whether connective tissue derivatives, cell membranes

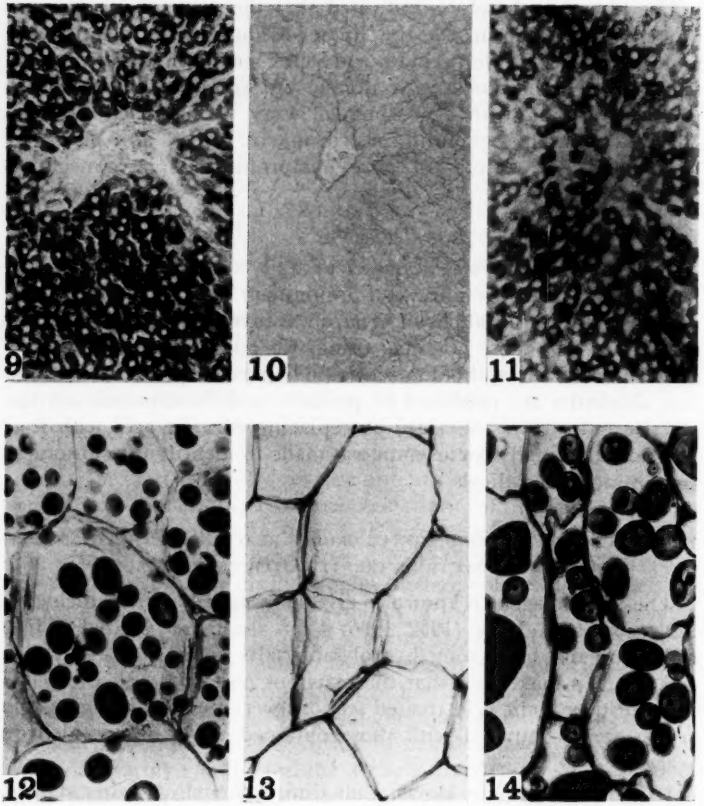


PLATE 3

Sections fixed in Orth's fluid, and stained with Schiff reagent after oxidation with periodic acid at room temperature or lead tetraacetate at various temperatures. The tissues were photographed with a green filter.

FIG. 9 — Liver. Periodic-acid-Schiff technic. Glycogen stains intensely.

FIG. 10 — Liver. Lead-tetraacetate-Schiff technic. The oxidation was carried out for 4 hours at *room temperature*. Under these conditions, the liver glycogen does not stain at all.

FIG. 11 — Liver. Lead-tetraacetate-Schiff technic. The oxidation was carried out for 3 hours at *70° C*. Under these conditions the liver glycogen stains intensely (compare Fig. 9).

FIG. 12 — Potato tuber. Periodic-acid-Schiff technic. Starch granules and cellulose in the cell membranes are stained.

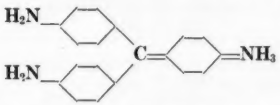
or secretion material were examined.[3] There was one exception, since the lens fibers gave a very intense reaction with benzidine and dianisidine, but not with the Schiff reagent. This exception did not affect the conclusion to be drawn from these experiments, namely, that all sites which stained with the Schiff reagent after periodic acid oxidation must have contained aldehyde groups, since they also reacted with benzidine and o-dianisidine.

The role of periodic acid in the periodic-acid-Schiff technic, is, therefore, to release or produce aldehyde groups – a conclusion in agreement with the original interpretation of Hotchkiss (1948).

THE NATURE OF THE RADICALS REACTING WITH PERIODIC ACID TO PRODUCE ALDEHYDE GROUPS

In order to identify the radicals capable of producing aldehyde groups on oxidation with periodic acid, a comparison was made with lead tetraacetate, another oxidant of similar though not identical properties. The behavior of 1,2–glycols, α-amino alcohols and α-hydroxy acids towards the two oxidants at room temperature is compared in Table 3.

[3]The fact that positive reactions were observed with benzidine and dianisidine after periodic acid oxidation has suggested a possible explanation for the reaction obtained by Arzac (1950b, 1952) by using colored basic fuchsin instead of fuchsin sulfurous acid. An examination of the structure of basic fuchsin



reveals that there are free amino groups which would be capable of undergoing a simple condensation reaction with free aldehyde groups in the same way as benzidine or dianisidine, and thus give a Schiff base. This possibility was also recognized by DeLamater, Mescon and Barger (1950), who found that formaldehyde and hydrolyzed desoxyribonucleic acid reacted with basic fuchsin to produce magenta compounds which could be precipitated.

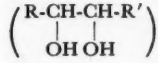
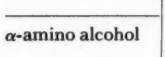
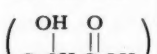
The reaction of the free amino groups of fuchsin would be an entirely different mechanism from the coupling with fuchsin sulfurous acid as indicated by the work of Wieland and Scheuing (1921).

FIG. 13 – Potato tuber. Lead-tetraacetate-Schiff technic. The oxidation was carried out for 4 hours at *room temperature*. Under these conditions, starch granules do not stain, but the cellulose membranes react as much as with periodic-acid-Schiff.

FIG. 14 – Potato tuber. Lead-tetraacetate-Schiff technic. The oxidation was carried out for 3 hours at *70° C*. Under these conditions the starch granules and cellulose membranes are stained (compare Fig. 12).

Note that glycogen and starch react differently from cellulose. This plate also demonstrates that temperature may influence the oxidizing properties of lead tetraacetate.

TABLE 3. COMPARISON OF LEAD TETRAACETATE AND PERIODIC ACID AS OXIDANTS FOR PRODUCING ALDEHYDE GROUPS FROM VARIOUS RADICALS AT ROOM TEMPERATURE

Radical	Oxidant	Products	Number of Aldehyde groups produced	References
1,2-glycol 	$Pb(OAc)_4$	$RCH{=}O+R'CH{=}O+Pb(OAc)_2+2AcOH$	2	Criegee (1931, 1932, 1948) Karrer & Hirohata (1933) Baer, Grosheintz & Fischer (1939) Criegee & Buchner (1940)
	HIO_4	$RCH{=}O+R'CH{=}O+HIO_3+H_2O$	2	Jackson (1944)
α-amino alcohol 	$Pb(OAc)_4$	$RCH{=}O+R'CH{=}NH+Pb(OAc)_2+H_2O$ $R'CH{=}NH \rightarrow RC{\equiv}N$ Imine Nitrile	1	Bollinger (1936) Leonard & Rebenstorf (1945) Criegee (1948)
	HIO_4	$RCH{=}O+R'CH{=}O+HIO_3+NH_3$	2	Jackson (1944)
α-hydroxy acid 	$Pb(OAc)_4$	$RCH{=}O+CO_2+Pb(OAc)_2+2AcOH$	1	Criegee, Kraft & Rank (1933) Oeda (1934) Fleury & Bon-Bernatets (1936)
	HIO_4	No reaction	0	Fleury & Lange (1933)

From this comparison, it appears that oxidation with lead tetraacetate at room temperature and subsequent staining with the Schiff reagent should reveal the locations of 1,2-glycols and α-amino alcohols, as after oxidation with periodic acid. In addition, lead tetraacetate should produce positive reactions with the α-hydroxy acids which may be present.

Lead tetraacetate is soluble in a wide variety of solvents which include benzene, chloroform, tetrachloroethane, nitrobenzene and glacial acetic acid (Criegee, 1931, 1932), but Criegee and Buchner

(1940) found that the addition of sodium acetate and potassium acetate to the glacial acetic acid solution greatly accelerated glycol cleavage.

While this manuscript was being prepared, several reports have appeared on the histochemical use of lead tetraacetate. Crippa (1951) oxidized with 1% lead tetraacetate in glacial acetic acid for one-half to one hour at room temperature and found that many mucous elements of gastric epithelia subsequently gave reactions with the Schiff reagent, as after periodic acid oxidation. The same solution was used in this investigation prior to knowledge of Crippa's work, but the length of oxidation was extended to 4 hours, and studies were also carried out at elevated temperatures on a wide variety of organs. Glacial acetic acid (without addition of acetate) was chosen as the solvent to obtain mild conditions of oxidation. However, Shimizu and Kumamoto (1952) used 1% lead tetraacetate in a mixture containing 30 volumes of glacial acetic acid to 70 volumes of saturated aqueous sodium acetate. Jordan and McManus (1952) used 0.9% lead tetraacetate in a solution composed of 25% sodium acetate dissolved in a mixture of equal volumes of water and glacial acetic acid. Lhotka (1952) employed a saturated solution of lead tetraacetate in glacial acetic acid, which was 0.5 *M* with respect to potassium acetate. Shimizu and Kumamoto claimed that 1% lead tetraacetate in glacial acetic acid liberates only small amounts of aldehyde groups. This observation is not compatible with the results obtained in the present investigation, since after oxidation for 30 minutes at room temperature all periodic-acid-Schiff positive structures (except glycogen, starch and the central portion of the cartilage matrix) reacted with the Schiff reagent. More intense reactions were obtained after oxidation for 4 hours at room temperature.

It has been known that lead tetraacetate oxidation is very sensitive to changes in concentration, solvent and the addition of sodium acetate or potassium acetate. It was also shown in the present investigation that temperature has an important influence on the oxidation.

The histochemical results (Table 1) showed an overall similarity between the reactions obtained with the two technics. Wherever there was a periodic-acid-Schiff reaction, it was possible to obtain a lead-tetraacetate-Schiff reaction (Table 1). The converse was also true.

Kuhn and Brockmann (1935) indicated that lead tetraacetate does not oxidize the –C=C– bond to produce an aldehyde group, since this oxidant reacted with the 1,2–glycol groups of carotene glycol without affecting the double bonds. In view of the agreement between the periodic-acid-Schiff and lead-tetraacetate-Schiff technics, this can be taken as evidence that the periodic-acid-Schiff reactions

were not due to –C=C– bonds (at least in the paraffin-embedded material examined here). Further evidence for this conclusion was found in the fact that bromination of tissues to saturate double bonds prior to periodic acid oxidation (Lillie, 1952) did not change the distribution or intensity of the reactions.

On the other hand, there were minor differences in the intensity of the Schiff reactions when the periodic acid and lead tetraacetate oxidations were compared, and these differences allow the classification of the reaction sites in three categories (see Results). Since these differences were consistently reproducible, they may be indicative of differences in the chemical constitution of the reactive materials. On this basis, one may speculate that the more intense reactions with periodic-acid-Schiff than with lead-tetraacetate-Schiff are due to a high concentration of α-amino alcohol radicals in these locations, since periodic acid liberates *two* aldehyde groups from an α-amino alcohol group, while lead tetraacetate produces only *one* aldehyde group (Table 3). This may be the case for reticulin fibers and membranes, acrosomic material, etc.

Those reactions, which are stronger with lead-tetraacetate-Schiff than with periodic-acid-Schiff, may be due to the presence of α-hydroxy acids, since these radicals react with lead tetraacetate to produce aldehyde groups but not with periodic acid. Other types of reactive groups would have to be present also, since the reaction with periodic-acid-Schiff was not negligible. This might be the case for the mucous neck cells of the stomach and the deeper goblet cells of the colon (Fig. 5 and 6).

However, these differences in staining intensity may be merely a result of the differences in rate or ease of reactivity of the various compounds with the two oxidants, a fact which is clearly shown in the case of glycogen and starch, to be discussed in the next section.

In conclusion, the results obtained with lead tetraacetate add confirmation to the interpretation that positive periodic-acid-Schiff reactions are due to the presence of 1,2–glycols and α-amino alcohols.

THE SUBSTANCES WITH RADICALS CAPABLE OF REACTING WITH PERIODIC ACID TO PRODUCE ALDEHYDE GROUPS

The following section is an attempt to determine whether or not certain polysaccharides, mucopolysaccharides, mucoproteins and proteins should give positive periodic-acid-Schiff reactions in histological sections, and is based on a consideration of what is known at present about their chemical structure, as well as on the results obtained with the film tests.

Polysaccharides

Glycogen, starch and cellulose: These three polysaccharides are polymers of anhydroglucose units and, therefore, have 1,2–glycol

groups which account for their reactivity (Table 1, Fig. 9 and 12). The reaction of glycogen and starch but not of cellulose was prevented by treatment of the sections with saliva. Our standard lead-tetraacetate-Schiff technic (oxidation for 4 hours at room temperature) produced no reaction with glycogen (Fig. 10) or starch granules (Fig. 13), although under these conditions, positive reactions were obtained with cellulose and all other periodic-acid-Schiff positive structures (Table 1, column 3).

However, oxidation with lead tetraacetate for 1 hour at 50° C., and particularly 3 hours at 70° C. caused positive Schiff reactions of glycogen and starch (Fig. 11 and 14).

It therefore appears that glycogen and starch are much less reactive than cellulose towards 1% lead tetraacetate in acetic acid. This may be due to the fact that glycogen and starch contain mainly or entirely α-glycosidal linkages (Meyer, 1943), whereas cellulose contains the β-glycosidal linkage.

Mucopolysaccharides

Hyaluronic acid: The positive Hotchkiss spot tests obtained on hyaluronic acid by Hotchkiss (1948) and Jorpes, Werner and Aberg (1948) have introduced the now widespread idea that the hyaluronic acid present in tissues is periodic-acid-Schiff positive (Gersh, 1947; McManus 1948; Jorpes, Werner and Aberg, 1948; Glick, 1949; Arzac, 1950a; Stoughton and Wells, 1950; Pearse, 1951; Cavallero and Braccini, 1951; Friberg, Graf and Aberg, 1951; Schrader and Leuchtenberger, 1951, and others).

However, three different samples of purified hyaluronic acid gave negative reactions in the film test (Table 2; Glegg, Clermont and Leblond, 1951; Davies, 1952), a fact in agreement with the finding of Meyer and Fellig (1950) and Jeanloz and Forchielli (1951) that hyaluronic acid consumes little periodic acid. This can be explained by the fact that its glucuronic acid and N-acetyl-D-glucosamine units are linked together in such a manner that there are no free 1,2–glycol or α-amino alcohol groups. It is, therefore, concluded that the presence of hyaluronic acid in sections cannot be demonstrated by the periodic-acid-Schiff technic.

This technic was criticized by Jeanloz (1950) on the basis that "hyaluronic acid. . . which yields a strong positive reaction (in histological sections), consumes only a very small amount of periodic acid". This criticism is no longer tenable in view of the present demonstration that hyaluronic acid is unreactive in the film.

Chondroitin Sulfuric Acid and Cartilage: Cartilage contains chondroitin sulfuric acid, which is itself made up of units of D-glucuronic acid and N-acetylchondrosamine-6–sulfate. Meyer, Odier and Siegrist (1946, 1948) found that 1 mole of chondroitin sulfuric acid con-

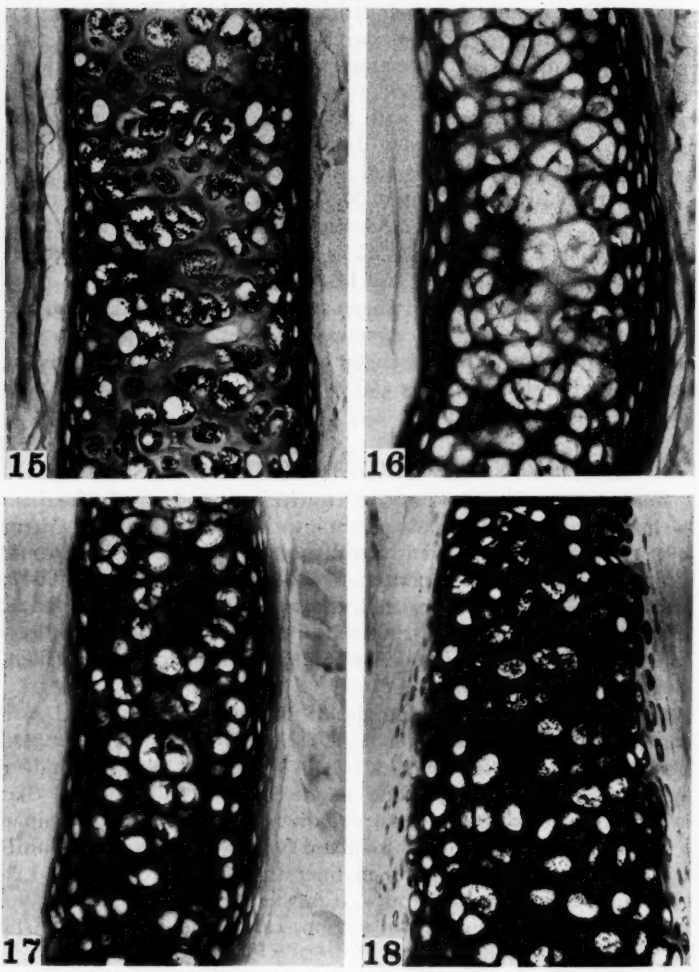


PLATE 4

Tracheal cartilage fixed in Orth's fluid, and stained with Schiff reagent after oxidation with periodic acid at room temperature or lead tetraacetate at various temperatures.

FIG. 15 — Periodic-acid-Schiff technic. The thin peripheral chondrogenic region reacts more intensely than the central portion of the matrix. Glycogen in the chondrocytes in both regions is intensely stained. Note the small elongated chondrocytes in the chondrogenic region.

sumed 4 moles of periodic acid in 2 hours and 8.6 moles in 36 hours. They concluded that, since there are about 120 monosaccharide units making up the molecule, the periodic acid consumption was very small, and could be accounted for by the terminal groups. Also, Jorpes, Werner and Aberg (1948) found that an insignificant amount of periodic acid was consumed by chondroitin sulfuric acid after 20 hours. Recently, Wolfrom, Madison and Cron (1952) found that, if the oxidation was prolonged for 60 hours, 1 mole of periodic acid was consumed for every 2 monosaccharide units. The histological test consists of an oxidation with periodic acid for 5–10 minutes only and therefore would be more comparable to the short experiments of Meyer *et al* (1948) in which the periodic acid consumption was low. Furthermore, periodic-acid-Schiff spot tests by Jorpes, Werner and Aberg (1948) and Dziewiatowski (1951) gave negative reactions for chondroitin sulfuric acid. Presumably, therefore, chondroitin sulfuric acid would have a sluggish or negative reaction in histological sections.

In the experience of the authors, treatment of sections of tracheal cartilage with testicular hyaluronidase (which has been shown by Meyer and Rapport (1950) to hydrolyze chondroitin sulfuric acid from hyaline cartilage) did not alter the positive periodic-acid-Schiff reaction of the matrix, although it removed the metachromasia and basophilia presumably due to chondroitin sulfuric acid.

This accumulation of data makes it unlikely that the periodic-acid-Schiff and lead-tetraacetate-Schiff staining of the cartilage matrix is due to chondroitin sulfuric acid. Incidentally, this staining is not due to glycogen either, since saliva treatment did not significantly decrease the reaction of the matrix, although it prevented the reaction in cartilage cells.

Furthermore, the thin peripheral chondrogenic region of tracheal cartilage stained more intensely than the central portion of the matrix with periodic-acid-Schiff (Fig. 15, Table 1). The same result was obtained after oxidation with lead tetraacetate for 4 hours at

FIG. 16 — Lead-tetraacetate-Schiff technic. The oxidation was carried out for 4 hours at *room temperature*. The thin peripheral chondrogenic region reacts more intensely than the central portion of the matrix, as with periodic-acid-Schiff (compare Fig. 15). However, the glycogen present in the chondrocytes is unstained under these conditions (compare Fig. 10).

FIG. 17 — Lead-tetraacetate-Schiff technic. The oxidation was carried out for 1 hour at *50° C*. Under these conditions the entire cartilage matrix (peripheral and central regions) is uniformly stained. Glycogen in the chondrocytes is stained faintly (see Table 1, Column 4).

FIG. 18 — Lead-tetraacetate-Schiff technic. The oxidation was carried out for 3 hours at *70° C*. The peripheral chondrogenic region is unstained, but its location is revealed by the presence of the small elongated chondrocytes (compare Fig. 16). The central portion of the matrix is more intensely stained than under any other conditions. Glycogen in the chondrocytes is also stained (compare Fig. 11).

room temperature (Fig. 16). Oxidation with lead tetraacetate for 1 hour at 50° C. caused the peripheral reaction to disappear completely, whereas the central portion of the matrix became much more intensely stained than when the oxidation was carried out at room temperature (Fig. 18). This suggests that the positive reactions of the tracheal cartilage may be ascribed to two substances with different locations and different properties.

It should be pointed out that Hisamura (1938) isolated a new polysaccharide from bovine tracheal cartilage and the basal septum of the whale after alkaline hydrolysis of the chondromucoid. This

TABLE 4. CARBOHYDRATE CONTENT

	Per cent	Kind	References
Serum globulin	1.82	Mannose and Galactose	(1)
Urease	12.5	Galactose	(2)
Egg albumen	1.71	Mannose	(1)
	3.67	Mannose	(2)
	1.8–2.82	Hexose	(3)
Thyroglobulin	2.3	Glucosamine,	
	4.22	Mannose and Galactose	(2)
	2.2	Glucosamine	(4)
	3.45	Carbohydrate	(5)
Mucin (glandular)	24.0	Galactose	(2)
	10.5	Glucosamine,	
	3.1	Hexose	(6)
	10–12	Glucosamine,	
	3.5	Hexose	(7)
	33.8	Galactose	(8)
Lens capsule	1.4	Glucosamine,	
	11.8	Hexose	(2)
	1.0	Hexosamine,	
	9–10	Hexose	(9)

(1) Sørensen and Haugaard (1933).
(2) Ujejski and Glegg (1952, Unpublished; the mucin was a commercial sample of gastric mucin).
(3) Neuberger (1938).
(4) Brand, Kassell and Heidelberger (1939).
(5) Salter (1940).
(6) Sublingual Mucin: Tanabe (1938).
(7) Submaxillary Mucin: Blix (1936).
(8) Gastric Mucin Polysaccharide: Gurin and Hood (1939).
(9) Pirie (1951).

polysacccharide was claimed to be a complex of equimolecular acetylchondrosamine and galactose. This polysaccharide component of cartilage may be responsible for the Schiff reaction after periodic acid or lead tetraacetate oxidation.

Heparin is a mucopolysaccharide, which may be sulfated to various degrees. The presence of three sulfate radicals per monosaccharide unit would block the groups capable of yielding aldehydes

with periodic acid (Jorpes, Werner and Aberg, 1948). The possibility of a less sulfated, more reactive form being also present in the body, might account for the fact that some mast cells stain with this technic (Leblond, 1950). It should be pointed out, however, that many mast cells are not reactive.

Chitin may be mentioned here, since its carbohydrate nature raises the possibility of its reactivity with periodic-acid-Schiff. It is a mucopolysaccharide consisting of anhydro-N-acetyl-glucosamine residues without free 1,2–glycol or amino alcohol groups (Meyer and Pankow, 1935; Meyer and Wehrli, 1937; Bergmann, Zervas and Silberkweit, 1931) and therefore should not give a positive reaction. Richards (1951, 1952) stated that *in vitro* chitin gives a negative reaction if dried, and indeed he observed little or no staining of the procuticle, which contains 25–60% chitin. However, he noted a positive reaction of the epicuticle, but he showed that this was not due to chitin.

TABLE 5. PERCENTAGES OF AMINO ACIDS (1)

Protein	Serine	Threonine	Hydroxylysine (2)
Gelatin	3.3	1.4	1.0
Casein	6–7	4.0	0.33
Trypsin	—	5.8	—
Pepsin	—	9.9	—
Edestin	0.7 (3)	3.1 (4)	0 (5)
Serum Globulin	—	6.8	—
Egg Albumen	7.6	3–4	0.09
Thyroglobulin	10.8 (6)	—	—
Lactogenic hormone (7)	6.5	4.8	—
Rat Liver Histone (8)	5.0	5.6	—

(1) Block and Bolling (1945), unless otherwise stated.
(2) Van Slyke, Hiller and MacFayden (1941), unless otherwise stated.
(3) Desnuelle (1938).
(4) Smith and Greene (1947).
(5) Desnuelle and Antonin (1946).
(6) Derrien, Michel, Pedersen and Roche (1949).
(7) Li (1949).
(8) Eadie and Leaf (1952).

Mucoproteins

Thyroglobulin: Hog thyroglobulin has been shown by Heidelberger *et al* (Table 4) to contain carbohydrate, and they identified one component as glucosamine. In the present investigation, 4.22% mannose and galactose and 2.3% glucosamine have been identified in a sample of beef thyroglobulin (Table 4) which was prepared and purified by the method of Derrien, Michel and Roche (1948). A 6μ thick film of this highly purified sample gave a very strong reaction which was of an intensity comparable to that obtained with the colloid, as observed in sections of thyroid follicles by Gersh (1947), McManus (1948) and Leblond (1950). This suggests that the positive staining of the colloid is due mainly to this substance. Furthermore,

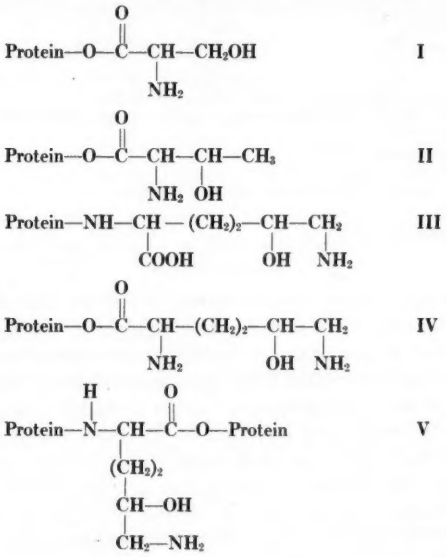
the mannose, galactose and glucosamine units respectively are presumably linked in such a way that their 1,2–glycol and possibly α-amino alcohol groups remain free and thus give a positive reaction.

Mucins (or *glandular mucus*): The presence of carbohydrates in mucins (Table 4) explains the reactions observed in histological sections. It may be recalled that, while many mucin accumulations react equally with periodic-acid-Schiff and lead-tetraacetate-Schiff (surface mucous cells of stomach, upper goblet cells of duodenum), there were several areas of stronger reaction with the latter method (mucous neck cells of the stomach, deeper goblet cells of the colon). This observation raises the possibility that α-hydroxy acid groups are present in the latter structures.

Lens capsule: Pirie (1951) has reported that the ox lens capsule contained 9–10% carbohydrate consisting of glucose and galactose, and 1% hexosamine. The ox lens capsule has also been isolated here and found to contain 11.8% hexose and 1.42% glucosamine. This large carbohydrate moiety is probably responsible for the intense periodic-acid-Schiff reaction observed in this structure (Leblond, 1950, Day, 1950).

Proteins

The presence of the hydroxy-amino acids, serine or threonine at the *ends* of the protein chains (I and II), or of hydroxylysine at any position in the chain (III, IV and V) would give rise to periodic-acid-Schiff reactions since the α-amino alcohol groups are exposed.



These arrangements must be infrequent since negative film tests were obtained with the proteins casein, pectinase, trypsin, pepsin, edestin, lipase, hyaluronidase, gelatin and rennin (Table 2), many of which are known to contain large amounts of serine and threonine (Table 5).

Whereas periodic acid could cleave structures I and II to leave an aldehyde group at the end of the protein chain, lead tetraacetate could not, since imines rather than aldehydes are produced from the amino-bearing carbon atom with the latter reagent (Table 3). However, the location of the amino and hydroxyl groups in hydroxylysine is the reverse of that in serine and threonine (Sheehan and Bolhofer, 1950), so that both the oxidants could produce aldehyde groups which could remain attached to the protein chain from structures III, IV and V. Periodic acid could, therefore, produce Schiff-positive products from proteins containing serine, threonine and hydroxylysine, if they were present in the arrangement specified above and their quantity was sufficient. Under the same conditions, lead tetraacetate would only produce Schiff-positive material from proteins containing hydroxylysine. A survey of the literature revealed that many proteins, such as casein, gelatin, edestin, trypsin, pepsin, serum globulin, egg albumen, thyroglobulin, myosin and the lactogenic hormone contain substantial quantities of serine or threonine, or both, but only small quantities of hydroxylysine (Table 5).

Thus, the lead-tetraacetate-Schiff technic should be more specific than periodic-acid-Schiff for demonstrating the location of polysaccharides in the presence of proteins. In this connection, it should be added that lead-tetraacetate-Schiff gave a much clearer background than periodic-acid-Schiff (Fig. 5 and 6). The light background seen with the former technic may be due to nonstaining of proteins.

In conclusion, the results obtained with the lead-tetraacetate-Schiff and other technics confirm the idea that positive periodic-acid-Schiff reactions are due to the presence of free 1,2–glycol and α-amino alcohol radicals. These radicals are mainly present in polysaccharides (free or combined with proteins as mucopolysaccharides and mucoproteins). Thus, the polysaccharides glycogen, starch and cellulose give very intense reactions. So do mucin, thyroglobulin and the lens capsule, which contain reactive sugar residues. Finally, the reactive structures of unknown chemical composition, such as reticulin fibers, brush borders, striated borders and the acrosomic system of spermatids must, by inference, also contain polysaccharides.

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TECHNICAL PROCEDURES FOR THE STUDY OF THE NUCLEOLONEMA

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ABSTRACT.—**In a recent paper[1] the authors have shown filamentous structures within the nucleolus of all cells. This structure was named the *nucleolonema.* In the present paper the technical procedures which prove their existence are described in detail.**

There are two groups: a) Silver impregnation procedures and iron-pyrogallic stain for fixed material; b) phase contrast microscopy, dark-field illumination and oblique transillumination for fresh material.

The suitable objects are mentioned in every case in order to obtain the best results.

TECHNICS

A. Silver Impregnation: All the silver impregnation technics can be used to demonstrate the *nucleolonema* if all the steps are properly timed and suitable fixatives are used.

The best impregnation of the nucleolonema not only in nerve cells but in all kinds of cells has been obtained by us employing Cajal's technic for Golgi apparatus and those neurofibrillar silver technics which use alcohol as the main fixative.

The nuclelonema can be selectively demonstrated by means of the following technic:

(1) Fixation in the following solution from 4 to 24 hours:

95–96% alcohol	60 ml.
Distilled water	30 ml.
Formalin	5 ml.
Diethyl ether	5 ml.
Uranium nitrate	1 g.

Four to six hours' fixation is enough for sympathetic and spinal root ganglia, while plant meristematic roots usually require from 20 to 24 hours.

(2) Impregnate for 36 to 48 hours in the dark at 37° C. in a 1.5 or 2% silver nitrate solution.

(3) Rinse in a mixture made up of equal parts of alcohol and distilled water from 3 to 5 minutes.

[1]ESTABLE, CLEMENTE and SOTELO, J. ROBERTO. 1951. Una nueva estructura celular: el nucleolonema. Instituto de Investigación de Ciencias Biológicas, Publicaciones, 1, 105–26.

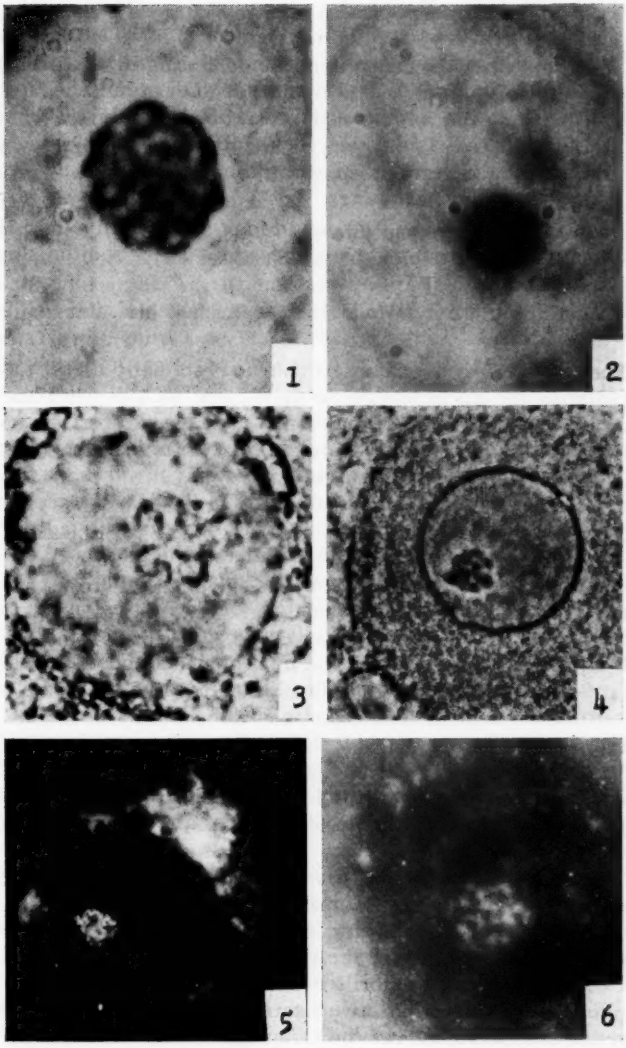
FIG. 1. Nucleolus of a neurone of cervical sympathetic ganglion of cat. Silver impregnation. 4500 ×.

FIG. 2. Nucleolus of a neurone of human medulla oblongata. Iron-pyrogallic technic. 4200 ×.

FIG. 3. Ovocyte of *Armadillidium nasatum* B.L. (terrestrian crustacea). Phase contrast 95 ×. Bright L. Spencer. 1425 ×.

FIG. 4. Ovocyte (early stage) of Armadillidium nasatum B.L. Phase contrast 55 ×. Bright L. Spencer. 1425 ×.

FIG. 5 & 6. Spermatocyte I of *Laplatacris Dispar* Rehn (Ortopthera) Darkfield illumination 1400 and 3330 × respectively.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(4) Reduce in the dark in the following solution for 5 to 10 hours:

Pyrogallic acid	1 g.
Commercial formalin (40%)	10 ml.
95–96% alcohol	20 ml.
Distilled water	70 ml.

(5) Wash out the reducing solution by immersing in distilled water for about 10 minutes. The amount of water should be 50 times greater than the volume of the tissue, or if less, several changes should be used. The tissues are then ready for sectioning with the freezing microtome, or embedding in celloidin or paraffin and subsequent sectioning.

(6) Tone the sections in a 0.2% solution of gold chloride in distilled water, rinse in pure water and fix in a 3–5% sodium thiosulfate solution for 10 minutes.

(7) Dehydrate, clear in xylene-creosote, xylene; blot and mount in balsam.

The sixth step is not necessary, but it is specially useful for discriminating the nucleolonema in some cases, such as when the nucleolonema is close to the chromosomes (during mitosis). The toning should be carefully checked by microscopic examination, otherwise it can be more prejudicial than useful.

The proportions of different substances in the fixative vary according to the cell or tissue employed. Ether offers distinct advantages when dealing with meristem, and in general with all vegetal cells, in spite of the shrinkage it produces. The fixative should also contain ether when an exclusive impregnation of the nucleolonema is desired regardless of whether the cells are vegetal or animal.

If an impregnation of both the nucleolonema and the Golgi apparatus is desired, ether should be eliminated from the fixative and the alcohol content should be under 50%.

Fixatives containing alcohol and uranium nitrate with or without formalin and lacking ether, are good for demonstrating the nucleolonema, the Golgi apparatus and the neurofibrils. It is very difficult to obtain a good impregnation of the three above-mentioned structures at the same time.

The factors which help to visualize the neurofibrils usually make the observation of the Golgi apparatus more difficult. However, this does not happen when dealing with nucleolonema and Golgi apparatus. A good fixative for the simultaneous demonstration of the nucleolonema and neurofibrils is a 10% uranyl nitrate solution in alcohol, and the most effective fixative for observing at the same time the nucleolonema and the Golgi apparatus is a mixture made up of 60–70 ml. distilled water, 20–30 ml. alcohol, 5–10 ml. formalin and 2 g. of uranyl nitrate.

The concentration and relative amounts of substances present in

the fixative deserve special attention.

If concentrated solutions of the fixative are used initially in order to insure a quick penetration, the solution should be gradually diluted later. This can be accomplished in three different ways:

(1) By adding water to it every hour.
(2) Transferring the pieces to a more dilute solution.
(3) Placing them in open containers to allow evaporation to bring about a gradual decrease in the concentration of the ether.

After having sufficient experience it will be possible for everyone to decide which dilution of the fixative is useful for adapting the general formula to each material.

We advise that one start working with sympathetic and spinal root ganglions in order to have a criterion about the results of the technic and the appearance of the nucleolonema. When other tissues are used, generally the thickness of the blocks should not be greater than 2 mm. It is of course understood that this limit can vary according to the nature of the tissue employed.

B. Iron-pyrogallic staining: The nucleolonema can occasionally be seen with certain stains (crystal violet for vegetal material) if the fixative is appropriate. Sometimes it can even be seen in ferric-hematoxylin slides but it cannot be clearly distinguished.

There is however a very useful staining technic by the use of which the nucleolonema can be seen as well in the interphasic nucleus as during mitosis.

This technic is based on the property possessed by pyrogallic acid of producing an insoluble blue-black coloration in the presence of a ferric salt when ferrous salts are added. This technic is performed as follows:

1) Fixation in 10% formalin, Sanfelice mixture (1% chromic acid, 16 ml.; formalin conc., 8 ml.; glacial acetic acid, 1 ml.) or even other fixatives containing chromic acid. Materials fixed over a long period of time can be used.
2) Paraffin embedding, frozen section, squashing, or sonic dispersion.
3) Immersion in a 3% iron alum solution, which acts as mordant, for at least 10 minutes.
4) Quick rinsing in distilled water.
5) Immersion in potassium ferricyanide for 30 seconds.
6) Adding, drop by drop, to the potassium ferricyanide a 2% pyrogallic acid solution (8 to 10 drops are sufficient). — Staining is immediate.
7) Rinsing.
8) Dehydration by alcohol, clearing with xylene-creosote, xylene, blotting and mounting in balsam.

All tissues (animal and plant) are suitable for the iron-pyrogallic stain. Motor neurons of the spinal cord of adult vertebrates, ovaries

of *Nothoscordum* plants, and meristematic roots of *Allium* provide the best results.

C. Post-vital staining:

Post-vital staining has been successful for the study of the nucleolonema in some cells which can be freed in the staining solution. Cells of *Ortoptheran* testis are a suitable material. The procedure is as follows:

1) Ortoptheran testes are dissected in saline solution (6.5 g. in 1000 ml.).
2) The testis tubules are placed in a watch glass and the staining solution (pyronin 2 g. in 1000 ml. of the saline solution) is dropped over them.
3) One or two tubules are placed on a slide and, with a cover glass, the tubes are squashed and the cells of the tubules spread out on the slide.
4) The cover glass is surrounded with paraffin in order to prevent evaporation, and after this, the slide is ready for observation.

If cells are well mixed with the staining solution the greatest part of them are stained, but often some cells are unstained because the spreading mass of cells is pushing the staining solution forward, and only the peripheral cells in contact with the solution are stained. In plant meristematic roots the procedure is similar but the roots must be in the staining solution for a long period of time. After 15 minutes, staining begins to color other cellular structures when animal tissue is used.

D. "In vivo" phase contrast darkfield illumination and oblique transillumination:

Different objects (animal and plant) may be used, but better results are obtained with spinal ganglion cells as well as ortoptheran spermatocytes and the ovaries of some terrestrian crustaceans *(Armadillidium).*

The material is mounted in saline solution and the cover slip is sealed with paraffin. Observations are made immediately after mounting in order to avoid post-mortem changes. In plants, best results are obtained in meristematic roots of *Nothoscordum, Allium* and *Vicia. Nothoscordum* is particularly useful because the meristematic cells may be freed from squashing.

WHOLE MOUNTS OF RABBIT LENS EPITHELIUM FOR CYTOLOGICAL STUDY

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ABSTRACT.—**The eye globe is cut equatorially just posterior to the equator and the anterior part fixed in acetic-acid:alcohol (1:3) for 24 hours. After washing, the lens is removed, hydrolyzed, and stained by the Feulgen technic. In the SO_2 wash solution, the lens epithelium is dissected free of the lens in one piece under a binocular microscope and laid on a cover slip, cell side down. It is flattened, after radial incisions, by absorbing water around the edge, and inverted on to a drop of glychrogel on a slide. Cell and nuclear structure are observed with the aid of phase contrast.**

There is much interest at present in radiation effects on the lens epithelium, the cells of which divide throughout life to give rise to new lens fibers. The lens epithelium is a single sheet of cells covering the anterior surface of the lens and lying between it and the acellular lens capsule. In the study of the epithelium, sections have the disadvantage that the lens, especially in mature animals, is notoriously difficult to cut. Poppe (1942) has described a method by which the epithelium can be stripped from the lens and mounted whole. His technic is admirable for histological study of the tissue, but the finer details of dividing nuclei are destroyed by the interval between the removal of the tissue from its blood supply and its fixation. The following method has been devised to allow for rapid fixation and to provide preparations suitable for mitotic counts and for more detailed cytological study. It has been used successfully with rabbits in this laboratory (see also von Sallmann, 1952) and could presumably be used for other animals.

The whole eye is removed immediately after the death of the rabbit or during deep fatal anesthesia. The posterior half of the globe is cut away with fine scissors just posterior to the equator, and the rest of the eye dropped into 3 parts absolute alcohol: 1 part glacial acetic acid. Fixation is for 24 hours, after which the eye can be stored in 70% alcohol.

After 1 hour in water, a needle is pushed into the posterior pole of the lens and the lens is pulled free of the iris and zonula Zinni, and dropped into 1 *N* HCl at 60°C. for 8 minutes. After rinsing in water and then in 1 *N* HCl at room temperature, the lens is stained in

Feulgen's reagent for 1 hour. The stain is replaced by two changes of SO_2 wash, in which the dissection is immediately carried out.

The lens is reimpaled on a needle pushed into the posterior pole, and freed of adhering iris tissue by wiping with the torn edge of wet filter-paper. Under a binocular microscope the nuclear bow may be seen as a pink line in the lens just posterior to the equator. With a small spatula the capsule is broken at this line and lifted and pulled forward until, with the epithelium on its inner surface, it is free of the lens. It tends to roll outwards, i.e., away from the lens. It is transferred, epithelium side down, to a drop of water. Three or four radial cuts are made through the equator and extending

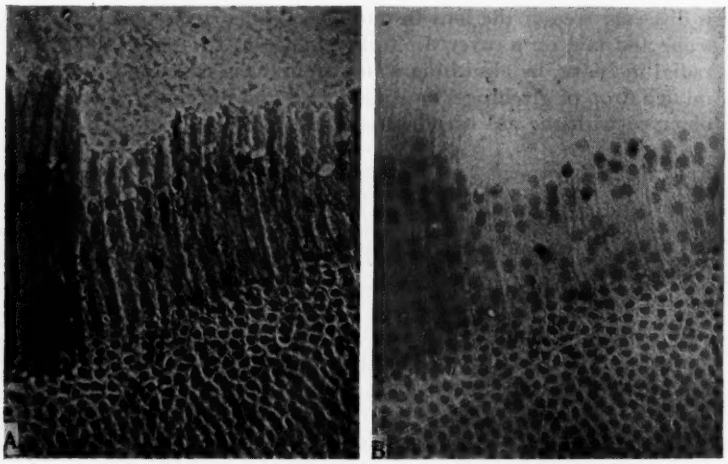

FIG. 1. — Equatorial zone of lens epithelium of a rabbit. Feulgen. ×425. a. Phase contrast. b. Without phase.

about ⅔ of the distance to the center. The capsule is then unrolled and flattened by absorbing water around its edge with filter paper. When all free water has been absorbed, but before the tissue begins to dry, the cover slip is inverted on to a slide bearing a large drop of warm glychrogel. (See Gatenby and Beams, 1950, p. 210).

The advantages of this mountant are, first, that the preparation does not have to be dehydrated before mounting and is consequently easily flattened. Secondly, the mountant is optically very suitable for phase contrast. It is quite permanent: slides made in this way 2 years ago are in excellent condition. Preparations should be kept level, without pressure, for 2–3 days after mounting; after this time they can be cleaned and stored in the usual way.

The advantages of phase contrast in conjunction with staining for observation of fixed whole mounts can be seen in the accompanying photomicrographs.[1] Fig. 1a shows a part of the equatorial zone of a normal rabbit taken by phase contrast at ×425. A band of young lens fibers, and on the left an overlying layer of slightly longer (older) ones, extend outwards from the edge of the epithelium. Fig. 1b is the same field taken without phase contrast. The stained nuclei of the fibers are visible but their outlines are not so clearly seen.

Fig. 2a shows a metaphase cell at some distance from the equator in the same preparation, photographed at ×1000 with phase contrast. Fig. 2b is the same field without phase contrast. The phase contrast

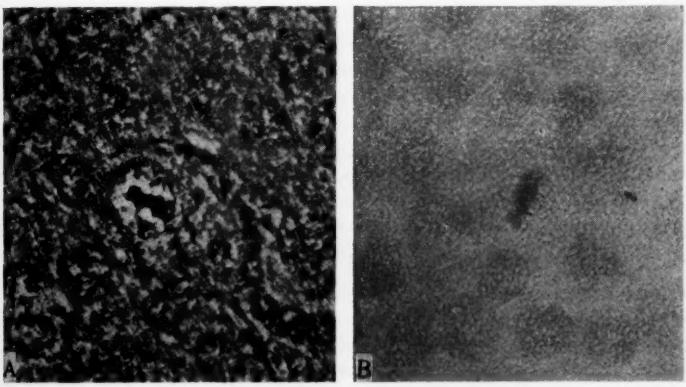

FIG. 2. — Metaphase cell in lens epithelium of a rabbit. Feulgen. ×1000.
a. Phase contrast. b. Without phase. (*Note: Fig. 2b oriented at angle of 90° with respect to 2a.*)

shows the spindle and cell boundary. In damaged cells the fragments seen by phase can be verified as nuclear or chromosomal by removing the phase plate and observing the Feulgen stain.

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[1] I am indebted to Dr. S. R. Pelc for taking the photomicrographs.

THE EFFECT OF FORMALDEHYDE ON TISSUE LIPIDS AND ON HISTOCHEMICAL REACTIONS FOR CARBONYL GROUPS[1]

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ABSTRACT.—**Histochemical and chemical evidence indicates that formaldehyde combines with unsaturated lipids at the double bond. The resulting complex contains a free carbonyl group which probably originates from the formaldehyde. The reaction occurs over a wide pH range, and takes place in the absence of oxygen or moisture. The reaction product is visualized by the Schiff reagent, and by the Ashbel-Seligman procedure. In the plasmal procedure, when performed on formalin-treated material, the reaction has the same significance as the pseudo-plasmal reaction, i.e. it denotes the presence of double bonds. The Ashbel-Seligman technic seems to be more sensitive to this complex than the Schiff reagent and shows it more markedly than it does the true plasmals and the atmospherically oxidized unsaturated compounds.**

The plasmal staining method has been shown to demonstrate two different classes of lipids because of two independently occurring reactions (Cain, 1949). The first reaction, known as the true plasmal, is due to splitting of acetalphosphatides (plasmalogens) by mercuric chloride or dilute acid, as shown by Feulgen and Bersin (1939). The second reaction, the *pseudo-plasmal,* was attributed to the production of carbonyl groups by atmospheric oxidation of C=C double bonds of unsaturated lipids (Verne, 1929; Gomori, 1942). Other procedures than the plasmal which have been proposed for demonstrating carbonyl groups, such as the phenyl-hydrazine (Bennett, 1940) or preferably the 2,4-dinitrophenylhydrazine reaction (Albert & Leblond, 1946) and the Ashbel-Seligman method (1949) visualize according to some authors the same compounds as Schiff's reagent (Herman & Dempsey, 1951), ketosteroids included. (Dempsey & Wislocki, 1944). Other authors believe them to stain mainly ketonic complexes such as the ketosteroids (Bennett, 1940; Ashbel & Seligman, 1949; Seligman & Ashbel, 1951).

This paper presents evidence which indicates that in formalin-treated tissues the carbonyl-demonstrating methods stain also a prod-

[1]This study was subvented by the Judah L. Magnes Fellowship.

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uct of combination between formaldehyde and unsaturated lipids, and that the formaldehyde-lipid complex contains a new carbonyl group which is probably derived from the formaldehyde.

I. Histochemical Procedures

Materials and methods: Fresh (unfixed) smears of guinea pig adrenal, brain and bone marrow, made as three parallel strips on the same slide, were used. No counterstaining was done. The following histochemical reactions were used:

A – *Schiff staining:* immersion in Schiff's fuchsin sulfurous acid for 10 minutes. Longer exposures were avoided as a rule, in order to minimize the hydrolytic action of the reagent. Exposures for 2 hours were used in the bromination experiments, after which the excess bromine was removed by sodium bisulfite solution. The longer exposure was necessary in order to hydrolyze the carbonyl-bisulfite complex (Lillie, 1950). In some experiments the usual plasmal procedure was performed with unfixed sections, in which a 10-minute treatment with 1% mercuric chloride preceded the immersion in Schiff's reagent.

B – *The Ashbel-Seligman naphthoic-acid-hydrazide reaction:* as described except for certain minor modifications. We used a commercial diazo product (Nat. Aniline Company's Blue BNS salt), and shortened the duration of the alcoholic and distilled water washes.

C – *Formalin fixation:* The smears were used either unfixed, or treated with formaldehyde at different stages of the various procedures. Formaldehyde vapor for 30–60 minutes was used in most instances. The slides were placed in a closed Coplin jar containing a few milliliters of concentrated formalin at the bottom, care being taken that the solution did not touch the smears. In some experiments formaldehyde gas was used under anaerobic conditions, either by introducing the concentrated formalin solution into an evacuated desiccator containing the slides, or by removing the oxygen from the desiccator by a pyrogallol-sodium-hydroxide mixture. In certain experiments the formaldehyde gas was used dry by introducing it into a desiccator containing phosphorus pentoxide after passing it over silica-gel and phosphorus pentoxide. In some experiments the slides were treated in the dark.

In other experiments formalin was used as a 10% solution in distilled water buffered with phosphate to different pH levels, under anaerobic conditions by diluting the formalin in freshly boiled distilled water in screw-capped Coplin jars completely filled and tightly capped, or else by the addition of 0.5% pyrogallol or sodium thioglycolate to the formalin in boiled water solution.

In every instance the slides were rinsed in running water for 10–30 minutes after fixation.

D – *Acetaldehyde treatment:* in a few experiments the slides were immersed in pure liquid acetaldehyde at room temperature for 1 hour in order to compare the results with those obtained by formaldehyde.

E – *Blocking of hydroxyl and amino groups:* was performed by acetylation in pure acetic anhydride at 60°C. for 24 hours. The procedure was controlled by a periodic-acid-treated section of a liver rich in glycogen. The procedure used was similar to that of Monné and Slautterback (1950) and differed from that of McManus and Cason (1950) in that no pyridine was used lest lipids be dissolved.

F – *Deamination:* was performed by immersing the slides in van Slyke's nitrous acid reagent (2 parts of concentrated sodium nitrite solution, 1 part of glacial acetic acid and 5 parts distilled water) for 24 hours at room temperature, in accordance with Monné and Slautterback's direction (1950).

G – *Carbonyl blocking agents:* it was found in preliminary experiments that the most suitable blocking agents for carbonyl groups were a molar solution of aniline hydrochloride (9.1 ml. of aniline, 8.1 ml. of concentrated hydrochloric acid, later diluted to 100 ml. with distilled water), and a 5% solution of phenylhydrazine hydrochloride (Lillie, 1952b). Blocking was performed by immersion of the slides in either of these solutions for 24 hours at room temperature. The first reagent was found more useful than the second, as it was found that phenylhydrazine, or its decomposition product (cf. Bennett, 1940) dissolved in fats like a Sudan stain. Since the dissolved phenylhydrazine was not washed out by water, it often interfered with the visualization of aldehydes at later stages.

H – *Bromination:* this was done by bromine vapor treatment for 4 hours at room temperature. The technic was similar to the technic of formaldehyde treatment. In order to remove excess bromine, bromination was followed by a 10-minute immersion in 5% sodium bisulfite.

I – *Oxidizing agents:* periodic acid oxidation was performed as prescribed by Lillie (1952b). The peracetic acid oxidation recently devised by Lillie and believed to selectively oxidize ethylenic linkages was employed (1952a). In some instances both reactions were continued for 24 hours in unsuccessful attempts to exhaust the reactive groups.

HISTOCHEMICAL EXPERIMENTS AND THEIR RESULTS

Fresh unfixed smears of adrenal and bone marrow were not stained by treatment with the Schiff reagent. Brain smears however, were colored pale red. After 2–4 days exposure to running tap water,[3]

[3]It should be noted that the tap water used in these experiments came from a municipal supply and was chlorinated, and that bacteria were not present on the slides.

smears of all three tissues were strongly colored by Schiff's reagent. These findings agree with previous reports (Verne, 1929a; Guyon, 1932; Verne, 1929b; Verne, 1936). Since leaving the dry slides in the air for the same period was followed by only slight coloration with Schiff's reagent, it seems probable that water catalyzes the oxidative reaction, or else allows oxidative enzymes to operate.

Exposure of the fresh smears to moist formaldehyde gas for as little as 30 minutes or to formalin solution in pH ranges 3–8 for 24 hours caused either the appearance or an increase in the intensity of the Schiff-staining. This reaction, i.e. the increased intensity of staining after formalin treatment (which will be referred to as the *formalin-effect*), also occurred in the absence of oxygen, in the presence of reducing agents, and in the absence of light. In the strongly acid range the formalin-effect was complicated by the additional effect of true plasmal hydrolysis. In order to circumvent this difficulty a prolonged (½–1 hour) hydrolysis by mercuric chloride or weak acid was followed by blocking of the liberated carbonyls, for only then could the slides be tested for the true formalin-effect.

The complex formed by the formalin-effect could be prevented from reacting with Schiff's reagent by aniline chloride and phenylhydrazine.

Results comparable to those obtained with Schiff's reagent were obtained with the Ashbel-Seligman technic. The latter method seemed to be more sensitive than the Schiff procedure to the formalin-effect, but less sensitive to either the true plasmal or the pseudoplasmal reactions. However, in the complete lack of moisture during the formaldehyde treatment the formalin-effect could not be detected with the Ashbel-Seligman procedure.

Experiments with smears treated with acetaldehyde instead of formaldehyde showed a similar staining of carbonyl groups by either the Schiff or the Ashbel-Seligman procedures.

In order to find out which molecular group was responsible for the formalin-effect, smears were subjected to various procedures prior to formalin treatment. The slides were then rinsed in running water for 30 minutes and stained with Schiff's reagent and in some experiments with the Ashbel-Seligman method. Procedures having no evident influence on the formalin-effect were: hydrolysis by mercuric chloride (followed by blocking of the carbonyls created), acetylation of hydroxyl and amino groups, and periodic oxidation (also followed by blocking of the carbonyls). On the other hand bromination, or to a lesser extent peracetic acid oxidation (followed by blocking of the carbonyls) abolished or markedly diminished the intensity of the formalin-effect. Prior deamination by van Slyke's reagent was followed by some increase in the intensity of the formalin-effect. This finding could be explained by the appearance of new ethylenic linkages which sometimes accompanies deamination

(Fuson, 1950). In fact peracetic acid oxidation after the deaminating procedure inhibited the formalin-effect.

II. Quantitative Chemical Procedures

Procedure: Linoleic acid in quantities of 1–2 g. (Fisher Sci. Co.–pure) was weighed accurately into small centrifuge tubes. A concentrated solution of formaldehyde (Merck, reagent, neutral) was diluted to a known strength (10–50% of the original concentration) and kept in a well stoppered container. Accurately measured amounts of the formaldehyde solution (2–10 ml.) were added to the linoleic acid, well stoppered, and put into a mechanical shaker for 18–24 hours. A control tube which contained the same amount of linoleic acid and distilled water instead of formaldehyde, was put simultaneously into the shaker.

At the end of the shaking, the tubes were centrifuged. The two liquid phases were pipetted off separately, and aliquots of each were taken for testing. The watery solutions were tested for the amount of formaldehyde, (using Romijn's iodometric procedure, Leffmann, 1923), and compared with a sample of the original formaldehyde solution. The linoleic acid phases were tested for their iodine combining capacity, a fresh aliquot from the original bottle serving as an additional control (using the technic described by Gattermann, 1948).

Results of the Chemical Procedures

At the end of the period of shaking it was quite clear that the linoleic acid which had been in contact with formaldehyde was more orange-yellow and less greenish than the linoleic acid in contact with distilled water. The iodine-combining capacity of the formaldehyde-treated linoleic acid was reduced by 1–2% as compared with the controls. Correspondingly, 1–2% of the total amount of formaldehyde disappeared from the solution which was in contact with the linoleic acid. When calculation was based on the assumption that one molecule of formaldehyde will block a double bond (compared to two atoms of iodine), it was found that less than one double bond disappeared together with the disappearance of two molecules of formaldehyde. The relationship between the disappearance of formaldehyde from the solution and the blocking of double bonds could not be accurately determined as the experimental error in determining these small changes was as high as 20% of the differences with the methods used.

Discussion

The histochemical experiments indicate that formaldehyde causes some lipids[4] to become both Schiff and Ashbel-Seligman positive.

[4]There is ample evidence in the literature indicating that the substances which are stained by the plasmal and Ashbel-Seligman procedures are lipids.

It is obvious that the reaction is not due to atmospheric oxidation, as anaerobic conditions do not prevent it. While an atmospheric oxidative reaction of unsaturated lipids probably does occur in formalin solutions, the formalin-effect might be considered as a competing reaction for the double bonds. The reaction seems to occur over a wide range of pH, at room temperature, and is independent of the presence of light, and as far as the Schiff procedure is concerned, the effect is not dependent on the presence of moisture. This last point seems to be important because the pseudo-plasmal reaction, with which the formalin-effect was confused until now, obviously depends very much on the presence of moisture. As the effect is not oxidative in nature, it seems plausible to suppose that the new carbonyl group selected by the reagents is derived from the formaldehyde. Furthermore, as the effect is also given by acetaldehyde, the assumption that the new carbonyl group is supplied by the aldehyde is strengthened.

There is evidence in the literature that formaldehyde combines with different radicals of proteins, such as the amino, amido, imino, guanidino and hydroxyl groups, peptide linkages, and the carboxyl, sulfhydryl and aromatic compounds (French & Edsall, 1945; Fraenkel-Conrat et al., 1945). In none of these reactions so far reported does the carbonyl group of formaldehyde remain free. An exception to this is an attempt by Tomiyama (1935) to explain the formaldehyde reaction with primary amines by coordinate valencies, but even this viewpoint has been severely criticized by Levy and Silberman (1937). While it is probable that some or all of these reactions occur in the proteins and lipids of tissues treated by formaldehyde, none of them would result in the formation of new carbonyl groups. In fact, acetylation and deamination had no influence on the formalin-effect in our experiments. The only procedures which inhibited the effect were those which destroyed the double bonds. On another occasion it has been found that some purified lipids from brain failed to show the formalin-effect after they had been kept exposed to air for several months. These facts seem to indicate that the point at which formaldehyde combines with lipids to give a compound containing a free carbonyl is at the double bond.

A number of papers have dealt with the effect of formaldehyde on tissue lipids. Some authors reported that certain lipids change their solubility and partly disappear from tissue immersed in formalin. (Weil, 1929; Mladenovic & Lieb, 1929; Kimmelstiel, 1929; Brante, 1929). Halliday (1939) reported a definite drop in the iodine numbers of human liver lipids after formalin treatment. She supposed the drop was due to atmospheric oxidation. It is possible that the different solubility of formaldehyde-treated lipids is partly due to the formation of formaldehyde complexes. Halliday's findings may be considered as corroboratory evidence for our conclusions.

It appears that the plasmal reaction is actually due, as far as we know, to two classes of compounds, the plasmalogens which can be hydrolyzed to yield the respective aldehydes, and non-saturated lipids. The non-saturated lipids are usually shown in the plasmal technics by either of two mechanisms or by both: (a) oxidation of the double bond, (b) combination with formaldehyde.

The Ashbel-Seligman procedure apparently gives a stronger reaction with the formaldehyde complex than with the oxidized lipids. As the authors themselves stated, their reaction depended on prior formalin treatment. It seems probable that the positive staining these authors obtained with ketosteroids was at least in part, a positive reaction for the *non-saturation* of the same ketosteroids.

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A RAPID MARCHI TECHNIC[1]

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ABSTRACT.—**By varying the thickness of the nervous tissue immersed in chlorate-osmic-formalin staining fluid (Swank and Davenport, 1935) it was found that a section 1 mm. thick can be completely and adequately stained in 24 hours. Thicker sections require a proportionately longer time. The quality of the Marchi stain in the rapidly prepared section is as good as that in the material stained for 10 days although the background is slightly lighter in the latter preparations. This method can be used where time is an important element and is especially applicable to spinal cord, small animal brains, or portions of larger brains in which serial sections are not required.**

The various modifications of the Marchi technic for staining degenerated nerve fibers are very time consuming. There are occasions when it would be highly advantageous to the investigator to be able to obtain well stained sections of nervous tissue showing fiber degeneration within a short period of time. Sometimes whole research projects are halted pending the results of the Marchi staining of a single brain. At other times, it would be desirable to know whether there is fiber degeneration present in a large mass of tissue before the entire brain is placed in the considerable volume of fluid containing the expensive osmic acid. Because of these needs, an investigation was undertaken to determine the factors responsible for the 2 to 3 weeks required by some of the common Marchi technics (Mettler, 1932; Swank and Davenport, 1934 and 1935; Krieg, 1949; and others), the changes that could be made to accelerate the process and the possible limitations of such modifications. Other investigators, such as Anderson (1929), Stewart (1936), and Rasmussen and Peyton (1941), have altered the Marchi method in various ways to reduce the required period of time.

PROCEDURE

Five monkeys were used for this experiment. In each monkey lesions were placed at various levels of the cord; after two weeks the animal was sacrificed and perfused with 10% formalin and the central nervous system fixed in the same strength of formalin. Selected

[1]This investigation was supported by a grant from the Alphonso Morton Clover Research Fund.

segments of the spinal cord, the brain stem and the cerebellum were stained by the Swank and Davenport (1935) modification of the Marchi technic with excellent results. This material was used by one of us in an investigation of the pattern of localization within certain ascending fiber systems of the spinal cord (Yoss, 1952a and 1952b). However, since only selected levels of the spinal cords were required, sections of various thicknesses (1, 2 or more mm.) from adjacent areas of the cord were stained along with the conventionally stained sections but were removed from the chlorate-osmic-formalin mixture (Swank and Davenport, 1935) at regular intervals during the 10-day staining period. These specimens were embedded in paraffin, sectioned (50 μ) and compared with the spinal cord sections that remained in the staining fluid a conventional period of time.

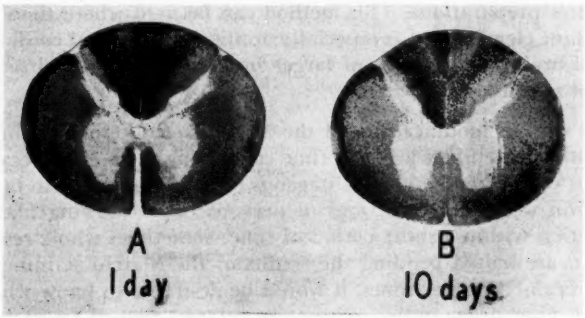

FIG. 1 — Upper cervical sections from the spinal cord of a monkey with spinal cord lesions (section at the 7th cervical segment of the right lateral and ventral white columns, the dorsomedial portion of the right dorsal column and the right dorsal root) and stained by the Marchi technic to show degenerated fibers. Section A was stained for 1 day; section B was stained the conventionally longer period of 10 days. Note the good quality of the stained degenerated fibers in both sections. ×8.

In addition to the monkey spinal cord, human material from the diencephalon was also prepared by this rapid method.

In one case, a slice of spinal cord from a monkey was placed, immediately following perfusion, in the staining solution for 24 hours, then washed, dehydrated and embedded in paraffin over a 10-hour period after which it was sectioned and mounted. The time which elapsed between sacrifice of the animal and microscopic examination of the spinal cord tissue was a little over 34 hours.

RESULTS

Spinal cord sections of 1 mm. thickness were entirely stained by the chlorate-osmic-formalin fluid after immersion for 24 hours.

Spinal cord slices of 2 mm. or of greater thickness were only partially stained at the end of such a period. They showed a clear, unstained area deep in the block of tissue approximately 0.5 mm. distance from the surface. In spinal cord sections 2 mm. thick but of smaller diameter (such as those through the thoracic cord of the monkey), the penetration of the stain was slightly greater (total penetration of 1.15 mm.) than that observed in spinal cord sections of larger diameter. In all the material studied (spinal cords from 5 different monkeys, a portion of the diencephalon of man), the degenerated fibers showed as well in the material immersed in the stain 24 hours as in that stained for 10 days. In both cases the degenerated fibers were easily identified and followed. Degeneration granules in the rapidly stained material are obviously of like character to those seen in sections stained for the longer period of time (Fig. 1). After 48 hours of immersion, material as thick as 2.5 mm. will be completely stained.

The only difference in appearance of spinal cord areas prepared by the rapid Marchi technic is in the color of the background of undegenerated myelinated nerve fibers. It was consistently found that the undegenerated fibers in sections so prepared appeared slightly darker brown than in material stained for a more prolonged period of time. The longer the material was in the staining solution (from 1 to 10 days) the lighter the background became. However, the difference in background was not great in any of the sections and did not interfere in any way with a study of the degenerated fibers (compare A and B, Fig. 1).

Discussion

Under certain conditions it is highly advantageous to obtain specimens of nervous tissue stained by the Marchi technic in as short a period of time as possible. Stewart (1936) speeded up the Marchi method by using frozen sections of nervous tissue but his procedure still required from 4 to 12 days. The conventionally long period of staining can be considerably shortened by the use of thin slices of nervous tissue. The period of time required for washing, dehydrating and embedding can likewise be shortened depending again upon the mass of the nervous tissue stained. It is suggested that this method is especially applicable to spinal cord, small animal brains, or material in which serial sections are not required. This method should be used only when time is an important element. Sections of nervous tissue of 1 mm. thickness will be completely and satisfactorily stained in 24 hours. It is to be stressed that the factor determining the minimal staining period is the thickness of the nervous tissue section; specimens thicker than 1 mm. will be stained if a correspondingly longer period of immersion in the staining fluid is employed.

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SYNTHETIC ORCEIN AS AN ELASTIC TISSUE STAIN

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ABSTRACT.—**A study was made of various synthetic orceins in an effort to determine the optimal conditions for their use in staining elastic tissue. A simple technic has been developed, based on a modification of the method originally worked out by Taenzer. Orcein is used as a 0.4% solution in 70% alcohol containing 1% HCl. Sections are counterstained with Mallory's borax methylene blue. The solutions are easily prepared and may be used for several months. Although several methods require staining in orcein from 2 to 24 hours, the present method requires staining for only 30 minutes. After several types of fixation some of the dye samples stained collagenous tissue to varying degrees, but this could be diminished by slightly reducing the strength of the staining solution. Differential staining of elastic tissue was particularly specific in tissues fixed in acetone, collagen in these preparations remaining practically unstained by any of the dye samples.**

The first to use orcein as an elastic tissue stain was apparently Taenzer, as reported by Unna (1890). It was originally used in alcoholic solution acidified with nitric acid. In later staining formulae (Unna, 1891 and 1894) the nitric was replaced by hydrochloric acid. Although orcein has thus been employed as a differential stain for elastic fibers for over 60 years, neither the chemical formula of this dye nor the nature of its apparently specific affinity for elastic fibers is understood. Various brands of synthetic orcein available during recent years have given somewhat variable results in elastic tissue staining. The present study was undertaken to examine several of these synthetic products and determine the effects of some of the variables in the staining procedure in an effort to define the optimal conditions for using these dyes in staining elastic tissue.

MATERIALS AND METHODS

Five samples of synthetic orcein were examined: three from Hartman-Leddon Co. (LOr–3, 5, 6) and two from National Aniline and Chemical Co. (NOr–2, 3). Of these batches, LOr–3 and 5 have recently been reported as very satisfactory elastin stains by Kornhauser (1952). The staining properties of these dyes were tested on paraffin embedded animal tissues fixed in Bouin, Zenker, 10% formalin, and

acetone. Spectral absorption studies of aqueous solutions of these dyes were done on a Beckman spectrophotometer.

After numerous tests, to be described below, the following procedure was found to give the most generally satisfactory staining results:

STAINING SOLUTIONS

A. Orcein ..... 0.4 g.
70% alcohol ..... 100 ml.
Conc. HCl ..... 1 ml.

B. Mallory's borax methylene blue for nuclear counterstain. (Mallory, 1938).
Stock solution:—Methylene blue ..... 1 g.
Borax ..... 1 g.
Distilled water ..... 100 ml.
For use:—dilute 1 part stock with 9 parts distilled water.

STAINING SCHEDULE

1. Bring the sections to water.
2. Stain 30 min. in orcein.
3. Rinse briefly with 70% alcohol.
4. Rinse with distilled water.
5. Stain 5 min. in diluted methylene blue solution.
6. Rinse with distilled water.
7. Differentiate and dehydrate in 95% alcohol containing about 0.5% of colophony (rosin). Keep the slide in *constant motion* and control the results under the microscope. Good results were obtained by differentiating about 2 minutes.
8. Complete the dehydration quickly with absolute alcohol.
9. Clear in xylene and mount in balsam.

Observations and Discussion

Elastic fibers are darkly stained by this procedure, although their color varies somewhat with different samples of dye: those from Hartman-Leddon Co. (LOr–3, 5, 6) stained the fibers a very dark purple, while samples from National Aniline and Chemical Co. (NOr–2, 3) stained the fibers a somewhat lighter reddish purple. Collagen was only faintly stained by the LOr group of samples, while the NOr group imparted to it a dusky pink color which did not seriously interfere with the recognition of fine elastic fibrils.

Spectrophotometric studies[1]*:* An attempt was made to determine whether or not variations in staining properties of the two groups of samples might be explained on the basis of chemical differences in

[1]Thanks to Mr. Nicholas Parente, chemist of the Biological Stain Commission.

the dyes themselves. Unfortunately chemical formulae and assay methods for orcein are not known. However, spectrophotometric studies should reveal any significant chemical differences between individual dye samples which might account for the variations in their staining properties. Each sample was examined in aqueous solution at a concentration of 1 mg. per 100 ml. in solutions varying in acidity from N/10 HCl to N/100 NaOH. Below pH 3.8 the absorption maximum for all specimens was between 510 and 515 mμ. No significant changes occurred in the region of pH 2.4 to 2.6, which as noted below is the region of transition from good or poor staining properties of the solution. When 70% alcohol was used as solvent, essentially similar results were obtained. Above pH 4 the absorption maximum of all specimens was between 576 and 582 mμ. The only apparent differences between individual samples were in the peak densities, which ranged from 0.553 to 0.719 and presumably indicated differences in dye concentration. The NOr samples showed the higher densities and the LOr samples the lower.

Two specimens (LOr–5 and NOr–3) tested by paper chromatography gave identical chromatograms except that the former was slightly fluorescent.

Effect of varying pH of staining solution: The orcein solution here recommended has a pH of about 1.2 to 1.3 (Beckman pH meter). By varying the amount of HCl the staining properties of solutions ranging from pH 0.2 to 3.1 were tested. Best staining of elastic tissue occurred between pH 1 and 2.4. Above pH 2.6 all specimens stained collagen increasingly red, confusing the recognition of elastic fibers. At pH 0.5 or lower the LOr samples gave a rather pale stain while the NOr samples still gave a satisfactory stain, although the collagen was tinted pink.

Various acids (1–5 ml. each) were tried in the above staining formula. Staining solutions were adjusted to pH 1–1.4 with nitric (Taenzer, cited by Unna, 1890; Kornhauser, 1943, 1945), hydrochloric (Taenzer, cited by Unna, 1891, 1894), sulfuric, perchloric, and trichloracetic acids. They all gave essentially indistinguishable staining results. With phosphoric acid, however, the pH did not go below 3 and the staining was poor.

These results indicate that it is immaterial which acid is used to acidify the staining solution, provided the resulting pH is between 1 and 2.4. Since the absorption spectrum of a given sample did not change appreciably over the range from pH 1 to pH 3.8 it would seem that the poor staining above pH 2.4 is probably due to some effect of the hydrogen-ion concentration on the elastic and collagenous fibers rather than on the stain itself.

Effect of dye concentration: Different authors have suggested various amounts of orcein in the staining solution, varying from 1%

(Taenzer; see Unna, 1890) to 0.4% (Kornhauser, 1943). In the present study of synthetic orceins the latter amount was found adequate and generally more satisfactory. More concentrated solution (up to 1%) of all samples stained collagen excessively, and more dilute solutions (down to 0.25%) stained elastic fibers too faintly. Since the above spectrophotometric studies show that the dye content may vary from sample to sample, some corresponding adjustment can be made in the preparation of the staining solution. Thus it was found that reddish staining of the collagen by NOr samples could be diminished by reducing the amount of stain to about 0.3% without affecting the elastic stain.

Effects of tissue fixation. This staining procedure has been applied to material fixed in Zenker, Bouin, 10% formalin, and acetone. Satisfactory differential staining of elastic tissue was obtained with each, although the most specific staining occurred in those fixed in acetone. Here the collagen was practically unstained, and with all dye samples even the finest elastic fibrils were stained intensely dark against an almost colorless background.

Summary

Several samples of synthetic orcein have been tested in staining elastic tissue. It was found that 0.4% solutions in 70% alcohol acidified to below pH 2.4 all gave satisfactory, although not identical, results. Staining contrast with individual samples could be slightly improved by adjusting the concentration of the staining solution in accordance with relative dye content or purity of sample.

Acknowledgment

I wish to acknowledge my indebtedness to Dr. Victor Emmel of the Department of Anatomy, University of Rochester, for his help during the progress of this work.

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A CONTROLLABLE CARMINE TECHNIC FOR PLANTS WITH SMALL CHROMOSOMES

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Received for publication May 15, 1952

ABSTRACT.—**Anthers of small chromosome plants (*Antirrhinum, Brassica, Capsicum,* etc.) were fixed 12 hours or longer at 0–3° C. in: ferric acetate in glacial acetic acid (sat. soln.), 1 part; absolute alcohol, 3 parts. They were transferred to: ferric acetate (sat. soln.) in 45% acetic acid, 3 parts; 45% acetic acid, 5 parts; 1% formalin (aq.), 2 parts, and allowed to remain 5–15 minutes at room temperature for mordanting. The amount of iron introduced into the specimens was controllable by the time in the mordanting fluid. After rinsing the specimen in 45% acetic acid and macerating in a drop of Belling's acetocarmine on a slide, a cover slip was applied followed by warming and pressing with blotting paper to flatten the pollen mother cells and expel excess stain. Preparations stored temporarily by sealing the edges of the cover slip with rubber solution were best made permanent by removing the cover slip after 1–2 days, dehydrating and mounting in euparal.**

Meiotic preparations in acetocarmine of plants with small chromosomes present greater difficulties than those of plants with large chromosomes. Good differentiation depends on four variables (Thomas, 1940) namely: composition of the prefixative, the duration of fixing, strength of acetocarmine and amount of iron used.

The present technic aims at greater control of differentiation by making the amount of iron introduced the only variable. Consequently the material is easier to handle and results are more uniform.

Fixation. Anthers at the desired stage are fixed in the following fixative:

1 part saturated solution of ferric acetate in glacial acetic acid.
3 parts absolute alcohol.

Preparations are best made after 12–16 hours fixation at a temperature of 0–3° C. However, excellent preparations have been made from material stored at 0—3° C. for up to seven months.

Mordanting. The mordanting solution is made as follows:

3 parts saturated solution of ferric acetate in 45% acetic acid.
2 parts 1% formalin (aq.)
5 parts 45% acetic acid.

Formalin is used as a hardening agent, but it may also have a mordanting effect as found with basic fuchsin (DeLameter, 1948).

Anthers are removed from the fixative a few at a time and placed in the mordanting solution at room temperature. The amount of iron needed depends on the material and is controlled by the time in the mordanting solution. *Solanum* and *Capsicum* species require about five minutes while *Pyrus* and *Malus* species need twice to three times as long before sufficient iron is introduced. The length of fixation also has to be considered, the longer the time in the fixative the shorter the period of mordanting. After long storage it may be omitted altogether. In general, stages prior to metaphase require a slightly longer period of mordanting.

Staining. After pretreatment, anthers are removed, rinsed quickly in 45% acetic acid, and placed in a drop of full strength acetocarmine. Should the material tend to overstain, due to overmordanting, a drop of 45% acetic acid to the drop of stain will bring the staining under control.

The anthers are teased with a *plated* needle and macerated with the end of a bone needle holder. Anther wall debris is removed before lowering the cover slip, and the drop of stain used should be only sufficient to reach the edges of the cover slip. The slide should be heated gently until a thin black line of precipitate develops at the edges of the cover slip, and while warm the cover slip pressed under a thickness of blotting paper. Due to the hardening effect of the formalin, pressure after heating seems to give better results than pressure before heating.

Preparations can be kept temporarily by ringing the cover slip with rubber solution and storing at 0–3° C. when not in use. They can be kept up to three days after which the cytoplasm darkens slightly.

Permanent preparations are best made after storing temporary preparations for 1–2 days. Storing seems to eliminate shrinkage of the cytoplasm when the cover slip is removed and gives better differentiation. La Cour's (1947) modification of Buck's (1935) method for making permanent acetocarmine preparations gives good results although it is best to omit the 10% acetic acid and float the cover slip off in 1:3 acetic alcohol. If the cytoplasm has darkened during

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All photographs are at a magnification of × 2000

Fig. 3 and 4 were taken from permanent preparations.

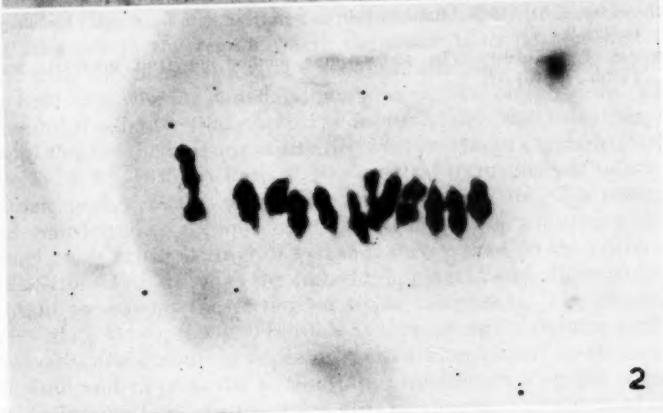
FIG. 1. *Pyrus communis* (2n–34) 17 bivalents.

FIG. 2. *Capsicum annum* (2n–24) 12 bivalents.

FIG. 3. *Lycopersicon esculentum* (2n–24) 12 bivalents.

FIG. 4. *Lycopersicon esculentum* (2n–24) Normal separation of 12 bivalents at Anaphase I.

1


2

3

4

storage then 45% acetic acid appears to be a better fluid to remove the cover slip, as this slightly destains and clears the cytoplasm. Permanent preparations of tomato pollen mother cells are still excellent after two years.

ACKNOWLEDGMENT

I wish to thank Mr. L. F. La Cour for his advice and criticism.

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NOTES ON TECHNIC

A Rapid Method in Section Mounting

On occasion, valuable tissue is lost because of undue hardness or faulty sectioning of tissue prepared by the paraffin method. A modification in the method of mounting has been used which serves to reclaim tissue which otherwise would have to be discarded. The method is also useful in classroom work where time is at a premium.

The sections are cut in the usual manner and collected in ribbons on tablet paper. The slides are cleaned in detergent solution or with *Bon Ami* cleaner. They are then dried and coated with Haupt's gelatin adhesive in the region where the tissue is to be mounted by spreading this material with a glass applicator rod. The rod should be held crosswise to and flat upon the surface of the slide. The amount of adhesive thus applied is approximately one half drop, or about double the amount ordinarily used in Haupt's technic (Stain Techn., **5**, 97; 1930). A drop of 40% neutral formaldehyde solution is then applied to this area. The section to be mounted is selected and transferred to the surface of the formaldehyde solution with a camel's hair brush. A drop of xylene is then placed on the center of the section. This dissolves the embedding paraffin and allows surface tension to expand the section of tissue completely. The reaction takes place very quickly. The slide is then pressed carefully with a lintless blotter (bibulous paper or smooth filter paper) to eliminate the fluid and to press the section into firm contact with the coagulated adhesive. Care must be taken that no slipping movement of the paper relative to the slide occurs. Coarse blotters cause distortion, often cling to the sections, and draw away parts or all of the sections. Before drying occurs, the slide is placed in the xylene jar of the staining series, because drying in air causes air inclusions in the tissue. The slides are then run down and stained in the usual manner.

This method is limited in its application to single mounts or multiple mounts in which orientation of one section to others is unimportant, since the sections drift independent of each other after application of the xylene to the tissue on the formaldehyde solution. Torn tissue will lead to the same complications as multiple sections. Wrinkled or compressed sections are expanded to conform with dimensions of the tissue in the block and apparently complete structural reorganization of the components is effected. No heating is required in this method, nor is any waiting time involved. Cleaning excess adhesive from stained slides is facilitated since it is not air dried. — A. J. Speece, Jr., *Dept. of Zoology, University of Texas, Austin, Tex.*

Dithioxamide for the Simultaneous Demonstration of Preformed Calcium and Alkaline Phosphatase

This communication is prompted by the report of J. Molnar on the use of rhodizonate in enzymatic histochemistry which appeared in a recent issue of Stain Technology.[1] In the case of alkaline phosphatase the calcium phosphate precipitate obtained at the sites of phosphatase activity is first converted into lead phosphate and then stained a brown-black shade with rhodizonate.

Several years ago the influence of experimental renal damage on histochemically demonstrable phosphatase and lipase activity was studied in the rat kidney.[2] Since calcium was deposited in some of the damaged kidneys, it became desirable to visualize *preformed* calcium which could not be distinguished in sections prepared for the demonstration of phosphatase activity. For this purpose, use was made of dithioxamide (rubeanic acid) — Eastman Kodak Co. #4394. Cobalt forms complex salts of rubeanic acid which are insoluble in water or diluted mineral acids and appear as intensely colored yellowish-brown precipitates.[3] The following technic was satisfactory:

Appropriately fixed tissues are prepared for phosphatase in the usual manner. Sections are covered with a 0.25% solution of celloidin in equal amounts of absolute alcohol and ether and placed for 15 minutes in a 2% solution of cobalt nitrate. After washing in tap water they are immersed for 1 minute in a dilute solution of ammonium sulfide. This stains most of the preformed calcium deposits black. After washing in tap water, slides are brought into the incubation mixture used for demonstration of alkaline phosphatase activity. A few drops of dilute ammonium sulfide are added to this mixture. The slides are then incubated at 37° C. as long as desired. After washing, they are placed for 15 minutes in a 2% cobalt nitrate solution. The formed cobalt phosphate is demonstrated by covering the slides with a saturated alcoholic solution of dithioxamide to which a few drops of ammonia water have been added. The preformed calcium, is stained black and contrasts well with the yellowish-brown, complex cobalt salt of rubeanic acid signifying phosphatase activity.

It is obvious that the sites of phosphatase activity can be demonstrated with any reagent that visualizes the formed calcium phosphate. While for most purposes the commonly used demonstration as black cobalt sulfide will remain the method of choice, the use of an organic reagent, like rhodizonate or dithioxamide, may be advantageous under certain conditions. — M. Wachstein, *Dept. of Pathology, St. Catherine's Hospital, Brooklyn, N. Y.*

[1] Molnar, J. 1952. The use of rhodizonate in enzymatic histochemistry. Stain Techn., **27**, 221–2.

[2] Wachstein, M. 1946. Influence of experimental kidney damage on histochemically demonstrable lipase activity in the rat. Comparison with alkaline phosphate activity. J. Expr. Med., **84**, 25–36.

[3] Feigl, F. 1937. Qualitative analysis by spot tests. Inorganic and organic applications. Nordemann Publishing Co., New York. See p. 84.

Bakelite Covers for Coplin Jars

Loss of caps of Coplin jars through breakage (and use as ash trays) has resulted in a large excess of jars in our laboratory. A satisfactory replacement has been found in the 53 mm. Bakelite screw cap for bottles and jars, which is a standard item in catalogs of scientific apparatus and supplies. The 53 mm. size fits most Coplin jars except the threaded type. It comes with either a foil or plain lining, but since the lining does not come into contact with the top of the jar, it may be removed if the cement which holds it becomes softened by such solvents as xylene or chloroform. The advantages of the Bakelite cover are that it is considerably less expensive than the glass cover, and it is practically unbreakable. — H. A. Davenport, *Northwestern University Medical School, Chicago, Ill.*

Method for Staining Tissue with Small Quantities of Stain

In order to stain tissues for long periods on standard slides with small quantities of dye fractions obtained by chromatography the following technic was found useful. Paraffin is placed on the free edge of a small spoutless glass beaker of 5 ml. capacity or less, either by dipping the edge of the beaker into melted paraffin or by painting on the paraffin with a warm brush. The beakers may be the 5 ml. size supplied regularly with each model G Beckman pH meter, or they may be prepared by cutting off the tops of glass vials. The stain is then collected in the beaker. The slide to be stained is heated slightly in an oven and then placed over the open end of the beaker

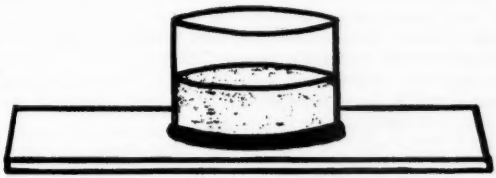

Fig. 1. — Beaker in place on slide.

in such a way that the tissue to be stained is in the beaker. The beaker and slide are pressed together for a few seconds until the paraffin hardens. The dye makes contact with the tissue when the slide and beaker are inverted (see figure). Several slides may be stained with the same small quantity of dye by transferring the beaker containing the dye from one slide to another. This technic has been used successfully with stains in aqueous and alcoholic solution. — Ralph L. Engle, Jr.[1] and Mary Jane Souders, *Dept. of Anatomy, Washington University School of Medicine, St. Louis, Mo.*

[1]American Cancer Society Research Fellow in Anatomy, recommended by the Committee on Growth of the National Research Council.

LABORATORY HINTS FROM THE LITERATURE

BOOK REVIEW

GOMORI, GEORGE. **Microscopic Histochemistry. Principles and Practice.** 5½ x 8½ inches. Cloth. 273 pp. University of Chicago Press. $6.00. 1952.

This book, by one of the leading specialists in histochemistry, is of value to anyone interested in this new branch of science. The author states that the subject matter of histochemistry is the identification and localization of chemical substances in tissues, on a cytological scale; but adds that in this book he uses the term in a restricted sense "to include only those methods in which the identifying chemical reaction is observed directly through the microscope, in tissues of which the architecture is not grossly altered."

The book is divided into two parts, the first (only about 25 pages in length) called Histochemical Methods in General, the second (nearly 200 pages) on Systematic Histochemistry. This latter section, comprising the main part of the book, discusses the histochemical demonstration of: inorganic substances, saccharides, lipids, proteins, amino acids, products of protein metabolism, prosthetic groups, etc., with about 90 pages devoted to the question of localizing enzymatic activity. The last mentioned subject is the author's specialty, and what he writes on it deserves very careful study.

The methods given throughout the book are not only described but are evaluated as to their reliability. The author points out that the subject is a new one, really beginning with the work of Lison in 1936; and that methods are not yet standardized. All students of histochemistry will agree with this; and will appreciate Dr. Gomori's critical evaluation of the methods now in use. — *H. J. Conn.*

HISTOCHEMISTRY

CRIPPA, A. 1951. **Sulla utilizzazione del tetracetata di piombo come ossidante in istochimica.** Soc. Ital. di. Biol. Sper., **27,** 599–601.

A histochemical technic for the cleavage of the 1,2–glycol radical utilizing lead tetraacetate is described. Routinely prepared paraffin sections are cleared in xylene and passed through a half-and-half mixture of xylene and glacial acetic acid into pure glacial acetic acid. The sections are then oxidized in a 1% solution of lead tetraacetate in glacial acetic acid. The period of oxidation is determined by trial. Following oxidation, the sections are washed by three changes of glacial acetic acid, again passed through the xylene-acetic acid mixture, and returned to pure xylene. The sections are then passed to water through graded alcohols, stained 20 min. in Schiff's reagent followed by the routine sulfite bath and running tap water. Sections are dehydrated, cleared, and covered. On examination, the sites of 1, 2–glycol linkages will be stained violet in a manner similar to the HIO_4-Schiff technic. — *John F. Lhotka.*

(Editor's note: This seems to have been the first report of histological use of lead tetraacetate.)

ZWEIFACH, B. W., BLACK, M. M., and SHORR, E. **Evaluation of tetrazolium as a histochemical index of adrenal cortical activity.** Proc. Soc. Exp. Biol. & Med., **76,** 446–54. 1951.

Tissues of adrenal cortex from rats and dogs were transferred into an ice-cold saline solution, and sectioned at 1–2 mm. thickness by hand at right angles to the surface in order to include all three adrenal cortical zones. The sections were rinsed in chilled saline and incubated for 1 hour at 37.5° C. in 5 ml. of a solution containing 3 parts of a 1% solution of 2, 3, 5–triphenyl tetrazolium chloride (TTC) to 1 part physiological saline stabilized at pH 7.2 with 0.1 *M* phosphate buffer. At the end of the incubation period, the solution was decanted and the tissue fixed in 10% formalin. Frozen sections of 5–15 μ were cut and mounted in glycerol. A series of photomicrographs are presented to show that reduction of TTC to red formazan is proportional to the zonal activity of the adrenal cortex. When incubation occurred in a medium containing an added substrate such as succinate, the intracellular staining pattern of formazon was identical with that which occurred without the added substrate. An accentuation of extracellular deposition of formazon was noted, however. — *Byron A. Myhre.*

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